

NOVEL HUMAN DICKKOPF-RELATED PROTEIN AND NUCLEIC ACID MOLECULES AND USES THEREFOR

Cross-Reference to Related Applications

5 The present application claims the benefit of prior-filed application PCT
Application No. PCT/US98/07894, filed April 16, 1998, which in turn claims the benefit
of prior-filed application U.S. Patent Application Serial No. 09/009,802, filed January
20, 1998. U.S. Patent Application Serial No. 09/009,802 claims the benefit of prior-
10 filed U.S. Patent Application Serial No. 60/071,589, filed January 15, 1998 and of prior-
filed U.S. Patent Application Serial No. 08/842,898, filed April 17, 1997. U.S. Patent
Application Serial No. 08/842,898 is a continuation-in-part of U.S. Patent Application
No. 08/843,704, filed April 16, 1997. The contents of each of the above-referenced
patent applications are incorporated herein by this reference in their entirety.

15 Background of the Invention

 Secreted proteins play an integral role in the formation, differentiation, and
maintenance of cells in multicellular organisms. For instance, secretory proteins are
known in the art to be involved in signaling between cells which are not in direct
contact. Such secreted signaling molecules are particularly important in the
20 development of vertebrate tissue during embryogenesis as well as in the maintenance of
the differentiated state of adult tissues. For example, inductive interactions that occur
between neighboring cell layers and tissues in the developing embryo are largely
dependent on the existence and regulation of secreted signaling molecules. In inductive
interactions, biochemical signals secreted by one cell population influence the
25 developmental fate of a second cell population, typically by altering the fate of the
second cell population. For example, the Wnt proteins are now recognized as one of the
major families of developmentally important signaling molecules in organisms ranging
from *Drosophila* to mice.

 The Wnt gene family encode a large class of secreted proteins related to the
30 Int1/Wnt1 proto-oncogene and *Drosophila* wingless ("Wg"), a *Drosophila* Wnt1
homologue, (Cadigan *et al.* (1997) *Genes & Development* 11:3286-3305). Wnts are
expressed in a variety of tissues and organs and are required for many developmental
processes, including segmentation in *Drosophila*, endoderm development in
Caenorhabditis elegans, establishment of limb polarity, neural crest differentiation,
35 kidney morphogenesis, sex determination, and brain development in mammals
(reviewed in Parr and McMahon (1994) *Curr. Opinion Genetics & Devel.* 4:523-528;
Cadigan and Nusse, *supra*).

Recent studies in diverse organisms have led to identification of several components of the Wnt signal transduction pathway in responding cells (Cadigan and Nusse, *supra*). Wnt signals are transduced by the Frizzled ("Fz") family of seven transmembrane domain receptors (Bhanot *et al.* (1996) *Nature* 382:225-230). The resulting signal leads to the activation of the cytoplasmic protein Dishevelled (Dsh) and stabilization of Armadillo/ β -catenin (Perrimon (1994) *Cell* 76:781-784). Negative regulators of the Wnt pathway include glycogen synthase kinase 3 (GSK3)/shaggy (Perrimon, *supra*), the tumor suppressor gene product adenomatous polyposis coli (APC) (Gumbiner (1997) *Curr. Biol.* 7:R443-436) and a novel protein, called Axin (Zeng *et al.* (1997) *Cell* 90:181-192). In the absence of a Wnt ligand, these proteins promote phosphorylation and then degradation of β -catenin, whereas Wnt signaling inactivates GSK3, thus preventing β -catenin degradation. As a result, β -catenin is translocated to the nucleus, where it forms a complex with TCF transcription factors and activates target gene expression (Cadigan and Nusse, *supra*). Deregulation of this pathway can lead to carcinogenesis (reviewed by Gumbiner, *supra*), emphasizing the long-recognized connection between Wnts, normal development and cancer. This connection has been further established recently with the identification the c-Myc proto-oncogene as a target of Wnt signaling (He *et al.* (1998) *Science* 281:1509-3512).

While the outcome of Wnt signaling may be influenced by multiple intracellular regulatory mechanisms, recent studies have identified several classes of secreted factors which can modulate Wnt action outside of the cell. These include Cerberus, a secreted Wnt inhibitor implicated in head development (Bouwmeester *et al.* (1996) *Nature* 382:595-601), and a family of proteins related to the extracellular domain of Frizzled. These Frizzled-related proteins ("FRPs") (Rattner *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:2859-2863), also known as secreted apoptosis-related proteins ("SARPs"), are encoded by several independently discovered genes including FrzA/FRP1, SDF5/FRP2, FrzB/FRP3, FRP4 and Sizzled (Melkonyan *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:13636-13641; Finch *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:6770-6775; Wang *et al.* (1997) *Cell* 88:747-766; Leyns *et al.* (1997) *Cell* 88:747-756; Mayr *et al.* (1997) *Mech. Dev.* 63:109-325; and Salic *et al.* (1997) *Development* 124:4739-4748). These proteins inhibit the ability of Xwnt8 to induce a secondary axis in frog embryos (for review see Zorn (1997) *Curr. Biol.* 7:R501-504), and are thought to compete for binding of Wnt ligands to the Frizzled receptors. Data on binding of certain FRPs to Xwnt8 (Wang *et al.*, (1997) *Biochem. Biophys. Res. Comm.* 236:502-504; and Leyns *et al.*, *supra*) and Wg corroborate this notion (Rattner *et al.*, *supra*).

It is now recognized that many of these families of signaling molecules have a dual role to play in both the development of an organism as well as in promoting or

conditions to a nucleic acid molecule consisting of nucleotides 470-2479 of nucleotide sequence shown in SEQ ID NO:1, to nucleotides 1-475 of nucleotide sequence shown in SEQ ID NO:4, or to nucleotides 1-600 of nucleotide sequence shown in SEQ ID NO:7, or hybridizes under stringent conditions to the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, to the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or to the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____. In another embodiment, the nucleic acid molecule is at least 500 nucleotides in length and hybridizes under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20 or a complement thereof.

Another embodiment of the invention provides an isolated nucleic acid molecule which is antisense to the coding strand of a Dkk nucleic acid or Dkk-related nucleic acid. Another embodiment of the invention provides an isolated nucleic acid molecules in a form suitable for expression of mRNA. In another embodiment, the isolated nucleic acid molecules are in a form suitable for expression of protein. In yet another embodiment, the isolated nucleic acid molecules are free from vector sequences.

Another aspect of the invention provides a vector comprising a Dkk nucleic acid molecule or Dkk-related nucleic acid molecule. In certain embodiments, the vector is a recombinant expression vector. In another embodiment, the invention provides a host cell containing a vector of the invention. The invention also provides a method for producing a Dkk protein or Dkk-related protein by culturing in a suitable medium, a host cell of the invention containing a recombinant expression vector such that a Dkk protein or Dkk-related protein is produced.

Another aspect of this invention features isolated or recombinant Dkk proteins and polypeptides or Dkk-related proteins and polypeptides. In one embodiment, an isolated Dkk protein has a signal sequence and a cysteine-rich region which comprises two cysteine-rich domains, and is secreted. In another embodiment, an isolated Dkk protein has an amino acid sequence sufficiently homologous to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:21. In a preferred embodiment, a Dkk protein has an amino acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:2. In another preferred embodiment, a Dkk protein has an amino acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:5. In another preferred embodiment, a Dkk protein has an amino acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:8. In another preferred embodiment, a Dkk protein has an amino acid sequence at least about 75% homologous to the amino acid sequence of SEQ ID NO:8. In another

indicator composition comprising a Dkk or Dkk-related protein having a biological activity, contacting the indicator composition with a test compound, and determining the effect of the test compound on the activity in the indicator composition to identify a compound that modulates the activity of a Dkk or Dkk-related protein.

5 Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

10 *Figure 1A-B* depicts the cDNA sequence and predicted amino acid sequence of human Dkk-3. The nucleotide sequence corresponds to nucleic acids 1 to 2479 of SEQ ID NO:1. The amino acid sequence corresponds to amino acids 1 to 350 of SEQ ID NO:2.

15 *Figure 2* depicts the cDNA sequence and predicted amino acid sequence of human Dkk-4. The nucleotide sequence corresponds to nucleic acids 1 to 848 of SEQ ID NO:4. The amino acid sequence corresponds to amino acids 1 to 224 of SEQ ID NO:5.

20 *Figure 3* depicts the cDNA sequence and predicted amino acid sequence of human Dkk-1. The nucleotide sequence corresponds to nucleic acids 1 to 1536 of SEQ ID NO:7. The amino acid sequence corresponds to amino acids 1 to 266 of SEQ ID NO:8.

Figure 4A-B depicts the cDNA sequence and predicted amino acid sequence of full-length human Dkk-2. The nucleotide sequence corresponds to nucleic acids 1 to 3687 of SEQ ID NO:20. The amino acid sequence corresponds to amino acids 1 to 259 of SEQ ID NO:21.

25 *Figure 5A-B* depicts the cDNA sequence and predicted amino acid sequence of murine Dkk-3. The nucleotide sequence corresponds to nucleic acids 1 to 2381 of SEQ ID NO:16. The amino acid sequence corresponds to amino acids 1 to 349 of SEQ ID NO:17.

30 *Figure 6* depicts a multiple sequence alignment of the amino acid sequences of hDkk-1 (corresponding the SEQ ID NO:8), mDkk-1 (having Accession No. AF030434), *Xenopus* Dkk-1 ("xDkk-1") (having Accession No. AF030433), hDkk-2 (corresponding to SEQ ID NO:21), hDkk-3 (corresponding to SEQ ID NO:2), mDkk-3 (corresponding to SEQ ID NO:17), chicken Dkk-3 ("cDkk-3") (having Accession No. D26311), and hDkk-4 (corresponding to SEQ ID NO:5). The alignment was performed using the
35 ClustalW algorithm as implemented in the GCG program PILEUP. The alignment provides information regarding the relationship between the Dkk proteins of the instant invention. Predicted signal peptides are underlined, N-glycosylation sites are indicated

by a thick bar, CRD-1 by an open box, CRD-2 by a shaded box. The proteolytic cleavage site within hDkk4 is indicated by an arrow.

Figure 7 depicts the cDNA sequence and predicted amino acid sequence of human Soggy. The nucleotide sequence corresponds to nucleic acids 1 to 928 of SEQ ID NO:13. The amino acid sequence corresponds to amino acids 1 to 242 of SEQ ID NO:14.

Figure 8 depicts the cDNA sequence and predicted amino acid sequence of murine Soggy-1. The nucleotide sequence corresponds to nucleic acids 1 to 835 of SEQ ID NO:26. The amino acid sequence corresponds to amino acids 1 to 230 of SEQ ID NO:27.

Figure 9 is a schematic diagram illustrating the Dkk and Dkk-related proteins of the instant invention. The figure depicts the structural domains of the human Dkks and Soggy. Signal peptides are indicated by darkened boxes. The cysteine-rich domains of a Dkk cysteine-rich region are depicted as CRD-1 and CRD-2. Branches indicate sites of N-glycosylation.

Figure 10 depicts a multiple sequence alignment of hSoggy-1 (corresponding to SEQ ID NO:14), murine Soggy-1 (corresponding to SEQ ID NO:27), hDkk-3 (corresponding to SEQ ID NO:2), and mDkk-3 (corresponding to SEQ ID NO:17). The alignment was generated as described in the legend to Figure 6. The alignment provides details regarding the relationship between the Dkk-3 and Soggy-1 proteins of the instant invention. Predicted signal peptides are underlined, N-glycosylation sites are indicated by a thick bar. CRD-1 and CRD-2 within Dkk-3 are indicated for reference by open and shaded boxes.

Figure 11 depicts a multiple sequence alignment of the carboxy-terminal cysteine-rich domains of hDkk-1, hDkk-2, hDkk-3, hDkk-4 with human colipase (having accession No. J02883). The carboxy-terminal cysteine-rich domains of the Dkk proteins are indicated by an open box. The alignment was generated using PILEUP (gap penalties of 12 for opening and 12 for extending). A minor adjustment was necessary since PILEUP inserts a single gap in hDkk-1 and hDkk-2 between Gly56 and Ser57, even with a gap opening penalty of 15. The conserved residues are indicated. The disulfide-bonding pattern typical for the colipase family and predicted for the Dkk family is indicated below the alignment.

Figure 12 is a schematic diagram depicting the relationship between the hDkk-3 nucleotide sequence (corresponding to SEQ ID NO:1) and those of RIG and RIG-like 7-1 (Accession Nos. U32331 and AF034208, respectively). Thick bars indicate regions of sequence identity between hDkk-3 and RIG or RIG-like 7-1 mRNAs. As between RIG and hDkk-3, there exists a short region of identity within the 3' untranslated regions of

the mRNAs when the mRNAs are aligned in reverse orientation. As between hDkk-3 and RIG-like 7-1, there exists a longer region of identity, however, RIG-like 7-1 lacks a signal sequence and, accordingly, is not predicted to be secreted.

5 **Detailed Description of the Invention**

The present invention is based on the discovery of novel molecules, referred to herein as Dkk protein and nucleic acid molecules, which comprise a family of molecules having certain conserved structural and functional features. The term "family" when referring to the protein and nucleic acid molecules of the invention is intended to mean
10 two or more proteins or nucleic acid molecules having a common structural domain and having sufficient amino acid or nucleotide sequence homology as defined herein. Such family members can be naturally-occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin, as well as other, distinct proteins of human origin or alternatively, can contain homologues of non-
15 human origin. Members of a family may also have common functional characteristics.

In one embodiment, a Dkk family member is identified based on the presence of at least one "cysteine-rich domain" in the protein molecule or corresponding amino acid sequence. As defined herein, a "cysteine-rich domain" refers to a portion of a Dkk protein (*e.g.*, hDkk-3) which is rich in cysteine residues. In a preferred embodiment, a
20 "cysteine-rich domain" is a protein domain having an amino acid sequence of about 45-85 amino acids of which preferably 10 amino acids are cysteine residues located at the same relative amino acid position as the cysteine residues in human Dkk-3 having SEQ ID NO:2 (*e.g.*, amino acid residues 147-195 of SEQ ID NO:2). In another embodiment, a "cysteine-rich domain" has 30-100 amino acids, preferably about 35-95 amino acids,
25 more preferably about 40-90 amino acids, more preferably about 50-80 amino acids, even more preferably about 55-75, 60-70, or 65 amino acids, of which at least about 3-20, preferably about 5-15, or more preferably about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids are cysteine residues.

A preferred Dkk protein of the present invention has a first cysteine-rich domain
30 ("CRD-1") referred to herein as an "amino-terminal cysteine-rich domain" or "N-terminal cysteine-rich domain" and a second cysteine-rich domain ("CRD-2"), referred to herein as a "carboxy-terminal cysteine-rich domain" or "C-terminal cysteine-rich domain". As defined herein, an "amino-terminal cysteine-rich domain" is a protein domain having an amino acid sequence of about 45-55 amino acids of which preferably
35 10 amino acids are cysteine residues located at the same relative position as the cysteine residues in an amino-terminal cysteine-rich domain of human Dkk-3 having SEQ ID

NO:2 (*e.g.*, amino acid residues 147-195 of SEQ ID NO:2). In another embodiment, an "amino-terminal cysteine-rich domain" has 30-70, preferably 35-65, more preferably about 40-60, and even more preferably about 46, 47, 48, 49, 50, 51, 52, 53, or 54 amino acids, of which at least about 3-20, preferably about 5-15, or more preferably about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids are cysteine residues. In a preferred embodiment, an amino-terminal cysteine-rich domain has the following consensus sequence: C - X(2) - D - X(2) - C - X(5) - C - X(8-13) - C - X(2) - C - X(6) - C - X(5) - C - C - X(4) - C - X(4) - C (SEQ ID NO:23). The consensus sequences described herein are described according to standard Prosite Signature designation (*e.g.*, all amino acids are indicated according to their universal single letter designation; X designates any amino acid; X(n) designates any n amino acids, *e.g.*, X (2) designates any 2 amino acids; and [LIVM] indicates any one of the amino acids appearing within the brackets, *e.g.*, any one of L, I, V, or M, in the alternative, any one of Leu, Ile, Val, or Met.)

As defined herein, a "carboxy-terminal cysteine-rich domain" is a protein domain having an amino acid sequence of about 80-85 amino acids of which preferably 10 amino acids are cysteine residues located at the same relative position as the cysteine residues in a carboxy-terminal cysteine-rich domain of human Dkk-3 having SEQ ID NO:2 (*e.g.*, amino acid residues 201-284 of SEQ ID NO:2). In another embodiment, a "carboxy-terminal cysteine-rich domain" has 65-100, preferably 70-95, more preferably about 75-90, and even more preferably about 81, 82, 83, or 84 amino acids, of which at least about 3-20, preferably about 5-15, or more preferably about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids are cysteine residues. In a preferred embodiment, a carboxy-terminal cysteine-rich domain has the following consensus sequence: C - X(4) - D - C - X(2) - G - X - C - C - X(8-10) - C - X - P - X(4) - G - X(2) - C - X(16-24) - C - X - C - X(2) - P - X(4) - G - X(2) - C - X(16-24) - C - X - C - X(2) - G - L - X - C - X(10-17) - C (SEQ ID NO:24).

A preferred protein of the present invention is a hDkk-3 protein (human Dkk-3) containing an amino-terminal cysteine-rich domain including about amino acids 147-195 of SEQ ID NO:2, having 10 cysteine residues, and a carboxy-terminal cysteine-rich domain including about amino acids 201-284 of SEQ ID NO:2, having 10 cysteine residues (the positions of the cysteine residues are depicted in Figure 6). In another embodiment, a hDkk-4 (human Dkk-4) protein contains an amino-terminal cysteine-rich domain including about amino acids 41-90 of SEQ ID NO:5, having 10 cysteine residues, and a carboxy-terminal cysteine-rich domain including about amino acids 138-218 of SEQ ID NO:5, having 10 cysteine residues (the positions of the cysteine residues are depicted in Figure 6). In another embodiment, a hDkk-1 protein (human Dkk-1)

cysteine-rich domain are located at the same relative position as the cysteine residues in a cysteine-rich domain of human Dkk-3 having SEQ ID NO:2. For example, as shown in Figure 6, human Dkk-4 has at least about 10 cysteine residues located at the same relative amino acid position as the cysteine residues in human Dkk-3 having SEQ ID

5 NO:2 (e.g., cys151 in Dkk-4, SEQ ID NO:5, is located at the same relative amino acid position as cys214 in Dkk-3, SEQ ID NO:2; cys156 in Dkk-4, SEQ ID NO:5, is located at the same relative amino acid position as cys219 in Dkk-3, SEQ ID NO:2; and cys157 in Dkk-4, SEQ ID NO:5, is located at the same relative amino acid position as cys220 in Dkk-3, SEQ ID NO:2). Similarly, as shown in Figure 6, Dkk-1 has at least about 10

10 cysteine residues located at the same relative amino acid position as the cysteine residues in human Dkk-3 having SEQ ID NO:2. As also shown in Figure 6, Dkk-2 has at least about 10 cysteine residues located at the same relative amino acid position as the cysteine residues in human Dkk-3 having SEQ ID NO:2. Table I sets forth at least 20

15 cysteine residues in each of hDkk-4, hDkk-1, and hDkk-2 which are located in the same relative position as 20 cysteine residues in hDkk-3.

Table I:

cysteine	aa position in hDkk-3	aa position in hDkk-4	aa position in hDkk-1	aa position in hDkk-2
1	147	41	85	78
2	153	47	91	84
3	159	53	97	90
4	168	63	111	100
5	171	66	114	103
6	178	73	121	110
7	184	79	127	116
8	185	80	128	117
9	190	85	133	122
10	195	90	138	127
11	208	145	189	183
12	214	151	195	189
13	219	156	200	194
14	220	157	201	195
15	231	166	210	204
16	241	176	220	214
17	265	194	237	231
18	267	196	239	233
19	273	202	245	239
20	284	218	263	256

The first 10 rows of Table I contain 10 cysteine residues that are included within the first, or amino-terminal, cysteine-rich domain of each of hDkks-3, -4, -1, and -2.

5 The last 10 rows of Table I contain 10 cysteine residues that are included within the second, or carboxy-terminal, cysteine-rich domain of each of hDkks-3, -4, -1, and -2.

Preferred Dkk proteins have more than one cysteine-rich domain, more preferably have at least two cysteine-rich domains and, thus, have a cysteine-rich region. As used herein, the term "cysteine-rich region" refers to a protein domain which includes

10 amino acid residues of which at least about 20 of the amino acids are cysteine residues. In another embodiment, a "cysteine-rich region" has preferably about 140-180 amino acid residues, and even more preferably at least about 135-175 amino acids of which at least about 10-30, preferably about 15-20, and more preferably about 16, 17, 18, or 19 of the amino acids are cysteine residues. In a preferred embodiment, a cysteine-rich region

15 is located in the C-terminal region of a Dkk protein. For example, in one embodiment, a hDkk-3 protein contains a cysteine rich region containing about amino acids 147-284 of SEQ ID NO:2, having 20 cysteine residues at the positions indicated in Figure 6. In another embodiment, a hDkk-4 protein contains a cysteine rich region containing about amino acids 41-218 of SEQ ID NO:5, having 20 cysteine residues at the positions

20 indicated in Figure 6. In another embodiment, a hDkk-1 protein contains a cysteine rich region containing about amino acids 85-263 of SEQ ID NO:8, having 20 cysteine residues at the positions indicated in Figure 6. In another embodiment, a hDkk-2 protein contains a cysteine rich region containing about amino acids 78-256 of SEQ ID NO:21, having 20 cysteine residues at the positions indicated in Figure 6.

25 In another embodiment, in addition to cysteine-rich domains, the cysteine-rich region contains a spacer region which separates the first and second cysteine-rich domains. As used herein, the "spacer region" refers to amino acid residues which are located between the first and second cysteine-rich domains of a cysteine-rich region and includes amino acid residues located C-terminal to the first cysteine-rich domain and N-

30 terminal to the second cysteine-rich domain. As defined herein, a "spacer region" refers to a protein domain of about 5-70 amino acids, preferably about 10-65 amino acids, more preferably about 15-60 amino acids, even more preferably about 20-55 amino acids, and even more preferably about 25-50, 30-45 or 35-40 amino acids. For example, hDkk-3 protein contains a spacer region of about amino acids 196-200 of SEQ ID NO:2;

35 hDkk-4 protein contains a spacer region of about amino acids 91-137 of SEQ ID NO:5; hDkk-1 protein contains a spacer region of about amino acids 139-181 of SEQ ID NO:8; and hDkk-2 protein contains a spacer region of about amino acids 128-175 of SEQ ID

NO:21. The spacer regions of hDkk-1, hDkk-2 and hDkk-4 are remarkably conserved in length (*e.g.*, the spacer region of hDkk-1 consists of 43 amino acid residues, the spacer region of hDkk-2 consists of 48 amino acid residues and the spacer region of hDkk-4 consists of 47 amino acid residues, suggesting that the close proximity of CRD-1 and CRD-2 is important in Dkk function. Accordingly, in one embodiment, the spacer region functions to spacially restrict the separation of CRD-1 from CRD-2.

In another embodiment of the invention, the Dkk protein has at least one cysteine-rich domain, preferably a cysteine-rich region, and a signal sequence. As used herein, a "signal sequence" refers to a peptide containing about 18-24 amino acids which occurs at the N-terminus of secretory and integral membrane proteins and which contains at least about 40-70% hydrophobic amino acid residues (*e.g.*, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, or proline). In another embodiment, a signal sequence contains at least about 8-34, 9-33, 10-32, 11-31, 12-30, 13-29, 14-28 amino acid residues, preferably about 15-27 amino acid residues, more preferably about 16-26 amino acid residues, more preferably about 17-25 amino acid residues, and more preferably about 18-24, 19-23, 20-22, or 21 amino acid residues, and has at least about about 50-65%, and more preferably about 55-60% hydrophobic amino acid residues (*e.g.*, alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, or proline). Such a "signal sequence", also referred to in the art as a "signal peptide", serves to direct a protein containing such a sequence to a lipid bilayer. For example, in one embodiment, a hDkk-3 protein contains a signal sequence of about amino acids 1-23 of SEQ ID NO:2. In another embodiment, a hDkk-4 protein contains a signal sequence of about amino acids 1-19 of SEQ ID NO:5. In another embodiment, a hDkk-1 protein contains a signal sequence of about amino acids 1-20 of SEQ ID NO:8. In another embodiment, a hDkk-2 protein contains a signal sequence of about amino acids 1-33 of SEQ ID NO:21. A preferred Dkk protein of the present invention is a human protein (*e.g.*, encoded by a nucleotide sequence corresponding to a naturally-occurring human gene).

Accordingly, one embodiment of the invention features a Dkk protein having at least one cysteine-rich domain, preferably at least one cysteine-rich region. Another embodiment features a Dkk protein having at least one cysteine-rich region, wherein the cysteine-rich region includes at least one cysteine-rich domain. Another embodiment features a Dkk protein having at least one cysteine-rich region, wherein the cysteine-rich region includes at least two cysteine-rich domains. Another embodiment features a protein or domain within a protein having 20, 30, 40, 50, 60, 70, 80, 90, 95, or 99% homology to a cysteine-rich domain of a Dkk protein of the invention (*e.g.*, hDkk-3, hDkk-4, hDkk-1, or hDkk-2).

Yet another embodiment of the invention features a Dkk protein having at least one cysteine-rich domain, preferably at least one cysteine-rich region and a signal peptide. Another embodiment features a Dkk protein having at least one cysteine-rich domain, preferably at least one cysteine-rich region and a signal peptide, wherein the
5 cysteine-rich region includes at least two cysteine-rich domains. Another embodiment features a Dkk protein having at least one cysteine-rich domain, preferably at least one cysteine-rich region and a signal peptide, wherein the cysteine-rich region includes at least two cysteine-rich domains and a spacer.

Yet another aspect of the invention features Dkk proteins having domains and/or
10 regions which are conserved among a subset of Dkk proteins but are not necessarily conserved among all Dkk family members. In one embodiment, a Dkk protein (*e.g.*, Dkk-3) has an "extended N-terminal region" which is extended in length as compared to, for example, the "N-terminal regions" of other Dkk family members (*e.g.*, Dkk-4, Dkk-1, and Dkk-2). As defined herein, an "N-terminal region" of a Dkk proteins consists of
15 amino acid residues found between the signal peptide and CRD-1 of a Dkk protein. Preferably, the first amino acid residue of an N-terminal region of Dkk is the first residue of a mature Dkk protein and the last residue of an N-terminal region of Dkk is the residue preceding the first cysteine residue of CRD-1. In a preferred embodiment, an N-terminal region is about 1-20 amino acid residues in length, preferably about 21-
20 30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, 101-110, 111-120, 121-130, 131-140, 141-150, 151-160 or more amino acid residues in length. In contrast, an "extended N-terminal region" is at least about 71-80, 81-90, 91-100, 101-110, 111-120, 121-130, 131-140, 141-150, 151-160 or more amino acid residues in length. For example, in one embodiment, a hDkk-4 protein includes an "N-terminal region" of about amino acids 20-
25 40 of SEQ ID NO:5 (21 amino acid residues in length). In another embodiment, a hDkk-1 protein includes an N-terminal region of about amino acids 21-84 of SEQ ID NO:8 (64 amino acid residues in length). In another embodiment, a hDkk-2 protein includes an "N-terminal region" of about amino acids 34-77 of SEQ ID NO:21 (44 amino acid residues in length). In another embodiment, a hDkk-3 protein has an
30 "extended N-terminal region" of about amino acids 23-146 of SEQ ID NO:2 (124 amino acid residues in length).

In another embodiment, a Dkk protein (*e.g.*, Dkk-3) has an "acidic C-terminal region" which includes amino acid residues found C-terminal to CRD-2 of a Dkk protein. Preferably, the first amino acid residue of an acidic C-terminal region is the residue
35 following the last cysteine of CRD-2 and the last residue of an acidic C-terminal region is the last residue of a Dkk protein. In a preferred embodiment, an acidic C-terminal region is about 65-66 amino acid residues in length and has about 27-25% acidic amino

acid residues (*e.g.*, glutamic acid or aspartic acid). In another preferred embodiment, an acidic C-terminal region is about 55-80 amino acid residues in length, preferably about 60-75 amino acid residues in length, and more preferably about 64-70 amino acid residues in length and has about 21-35% acidic amino acid residues, preferably about 23-33% acidic amino acid residues, and more preferably about 25-31% acidic amino acid residues. Preferably, an acidic C-terminal region is involved in protein-protein interactions. For example, in one embodiment, a hDkk-3 protein has an acidic C-terminal region from about amino acids 285-350 of SEQ ID NO:2.

Preferred Dkk molecules of the present invention have an amino acid sequence sufficiently homologous to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:21. As used herein, the term "sufficiently homologous" refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent (*e.g.*, an amino acid residue which has a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences share common structural domains and/or a common functional activity. For example, amino acid or nucleotide sequences which share common structural domains have at least about 40% homology, preferably 50% homology, more preferably 60%-70% homology across the amino acid sequences of the domains and contain at least one, preferably two, more preferably three, and even more preferably four, five or six structural domains, are defined herein as sufficiently homologous. Furthermore, amino acid or nucleotide sequences which share at least 40%, preferably 50%, more preferably 60, 70, or 80% homology and share a common functional activity are defined herein as sufficiently homologous.

As used interchangeably herein, a "Dkk activity", "biological activity of Dkk" or "functional activity of Dkk", refers to an activity exerted by a Dkk protein, polypeptide or nucleic acid molecule (*e.g.*, an activity on a Dkk responsive cell) as determined *in vivo*, or *in vitro*, according to standard techniques. In one embodiment, a Dkk activity is a direct activity, such as an association with a Dkk-target molecule. As used herein, a "target molecule" is a molecule with which a Dkk protein binds or interacts in nature, such that Dkk-mediated function is achieved. A Dkk target molecule can be a non-Dkk molecule or a Dkk protein or polypeptide of the present invention. In an exemplary embodiment, a Dkk target molecule is a membrane-bound protein (*e.g.*, a cell-surface receptor or "Dkk receptor") or a modified form of such a protein which has been altered such that the protein is soluble (*e.g.*, recombinantly produced such that the protein does not express a membrane-binding domain). In another embodiment, a Dkk target is a second soluble protein molecule (*e.g.*, a "Dkk binding partner" or "Dkk substrate"). In

such an exemplary embodiment, a Dkk binding partner can be a second soluble non-Dkk protein or a second Dkk protein molecule of the present invention. Alternatively, a Dkk activity is an indirect activity, such as a cellular signaling activity mediated by interaction of the Dkk protein with a second protein (*e.g.*, a Dkk receptor). As used
5 herein, the term "Dkk receptor" refers to a protein or protein complex, to which a Dkk protein, *e.g.*, human Dkk, can bind. A receptor can be a cell surface receptor, *e.g.*, a peptide, growth factor, or nuclear hormone receptor. Dkk receptors can be isolated by methods known in the art and further described herein. Interaction of a Dkk protein with a Dkk receptor can result in transduction of a signal from the cell surface to the nucleus.
10 The signal transduced can be, an increase in intracellular calcium, an increase in phosphatidylinositol or other molecule, and can result in, *e.g.*, in phosphorylation of specific proteins, a modulation of gene transcription and any of the other biological activities set forth herein.

In a preferred embodiment, a Dkk activity is at least one or more of the following
15 activities: (i) interaction of a Dkk protein with and/or binding to a second molecule, (*e.g.*, a protein, such as a Dkk receptor, a soluble form of a Dkk receptor, a receptor for a member of the *wnt* family of signaling proteins, or a non-Dkk signaling molecule, for example, a lipid included in a cell membrane); (ii) interaction of a Dkk protein with an intracellular protein *via* a membrane-bound Dkk receptor; (iii) complex formation
20 between a soluble Dkk protein and a second soluble Dkk binding partner (*e.g.*, a non-Dkk protein molecule or a second Dkk protein molecule); (iv) interaction with other extracellular proteins (*e.g.*, regulation of *wnt*-dependent cellular adhesion to extracellular matrix components); (v) binding to and eliminating an undesirable molecule (*e.g.*, a detoxifying activity or defense function); and/or (vi) an enzymatic activity. In yet
25 another preferred embodiment, a Dkk activity is at least one or more of the following activities: (1) modulation of cellular signal transduction, either *in vitro* or *in vivo* (*e.g.*, modulation, *e.g.*, antagonism, of the activity of members of the *wnt* family of secreted proteins or suppression of *wnt*-dependent signal transduction, for example suppression of Wnt 2b, Wnt3 and/or Wnt8-dependent signal transduction by hDkk-1 and/or hDkk-4);
30 (2) regulation of communication between cells (*e.g.*, regulation of *wnt*-dependent cell-cell interactions); (3) regulation of expression of genes whose expression is modulated by binding of Dkk (*e.g.*, hDkk-3) to a receptor; (4) regulation of gene transcription in a cell involved in development or differentiation, either *in vitro* or *in vivo* (*e.g.*, induction of cellular differentiation); (5) regulation of gene transcription in a cell involved in
35 development or differentiation, wherein at least one gene encodes a differentiation-specific protein; (6) regulation of gene transcription in a cell involved in development or differentiation, wherein at least one gene encodes a second secreted protein; (7)

regulation of gene transcription in a cell involved in development or differentiation, wherein at least one gene encodes a signal transduction molecule; (8) regulation of cellular proliferation, either *in vitro* or *in vivo* (e.g., induction of cellular proliferation or inhibition of proliferation as in the case of suppression of tumorigenesis (e.g., suppression of glial cell tumor growth, for example, glioblastoma growth)); (9) formation and maintenance of ordered spatial arrangements of differentiated tissues in vertebrates, both adult and embryonic (e.g., induction of head formation during vertebrate development or maintenance of hematopoietic progenitor cells); (10) modulation of cell death, such as stimulation of cell survival; (11) regulating cell migration; and/or (12) immune modulation.

As referred to herein, "differentiation-specific proteins" include proteins involved in the transition of a cell from the undifferentiated to the differentiated phenotype. For example, such proteins can be differentiation specific structural proteins or differentiation-specific transcription factors. Such differentiation-specific proteins are generally expressed at higher levels in cells which are making the transition from the undifferentiated to the differentiated phenotype (e.g., during embryonic development or during regeneration of mature tissue in the adult animal), or are expressed at higher levels in fully-differentiated or terminally-differentiated cells as compared to their undifferentiated counterparts. Also, as referred to herein, "differentiation-specific genes" include nucleic acid molecules which encode differentiation-specific proteins.

Accordingly, another embodiment of the invention features isolated Dkk proteins and polypeptides having a Dkk activity. Preferred Dkk proteins have at least one cysteine-rich region and a Dkk activity. In another preferred embodiment, the Dkk protein has at least one cysteine-rich region, wherein the cysteine-rich region comprises at least one cysteine-rich domain, and a Dkk activity. In another preferred embodiment, the Dkk protein has at least one cysteine-rich region, wherein the cysteine-rich region comprises at least two cysteine-rich domains, and a Dkk activity. In yet another preferred embodiment, a Dkk protein further comprises a signal sequence. In still another preferred embodiment, a Dkk protein has a cysteine-rich region, a Dkk activity, and an amino acid sequence sufficiently homologous to an amino acid sequence of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, or SEQ ID NO:21.

A preferred Dkk fragment comprises a carboxy-terminal cysteine-rich domain. In one embodiment, a Dkk fragment comprises a carboxy-terminal cysteine-rich domain and retains a biological activity of a Dkk protein. In yet another embodiment, a Dkk fragment lacks an amino-terminal cysteine-rich domain.

The human Dkk-3 cDNA, which is approximately 2479 nucleotides in length, encodes a protein which is approximately 350 amino acid residues in length. The

human Dkk-3 protein contains an N-terminal signal sequence and a cysteine-rich region comprising two cysteine-rich domains. A Dkk cysteine-rich region can be found at least, for example, from about amino acids 147-284 of SEQ ID NO:2. The hDkk-3 cysteine-rich region comprises an amino-terminal cysteine-rich domain from about amino acids 147-195 of SEQ ID NO:2 and a carboxy-terminal cysteine-rich domain from about amino acids 201-284 of SEQ ID NO:2. The human Dkk-3 protein is a secreted protein which further contains a signal sequence at about amino acids 1-21, 1-22, 1-23, or 1-24 of SEQ ID NO:2. Accordingly, a mature human Dkk-3 protein begins at about amino acid residue 22, 23, 24, or 25 of SEQ ID NO:2 and is about 329, 328, 327, or 326 amino acids in length. The prediction of such a signal peptide can be made, for example, utilizing the computer algorithm SIGNALP (Nielsen, *et al.*, (1997) *Protein Engineering* 10:1-6).

The human Dkk-4 cDNA, which is approximately 848 nucleotides in length, encodes a protein which is approximately 224 amino acid residues in length. The human Dkk-4 protein contains an N-terminal signal sequence and a cysteine-rich region comprising two cysteine-rich domains. A Dkk cysteine-rich region can be found at least, for example, from about amino acids 41-218 of SEQ ID NO:5. The hDkk-4 cysteine-rich region comprises an amino-terminal cysteine-rich domain from about amino acids 41-90 of SEQ ID NO:5 and a carboxy-terminal cysteine-rich domain from about amino acids 138-218 of SEQ ID NO:5. The human Dkk-4 protein is a secreted protein which further contains a signal sequence at about amino acids 1-17, 1-18, 1-19, or 1-20 of SEQ ID NO:5. Accordingly, a mature human Dkk-4 protein begins at about amino acid residue 18, 19, 20, or 21 of SEQ ID NO:5 and is about 207, 206, 205, or 204 amino acids in length. A preferred fragment of hDkk-4 comprises amino acid residues 134-224 of SEQ ID NO:5. In another embodiment, a preferred fragment of hDkk-4 consists of amino acid residues 134-224 of SEQ ID NO:5.

The human Dkk-1 cDNA, which is approximately 1536 nucleotides in length, encodes a protein which is approximately 266 amino acid residues in length. The human Dkk-1 protein contains an N-terminal signal sequence and a cysteine-rich region comprising two cysteine-rich domains. A Dkk cysteine-rich region can be found at least, for example, from about amino acids 85-263 of SEQ ID NO:8. The hDkk-1 cysteine-rich region comprises an amino-terminal cysteine-rich domain from about amino acids 85-138 of SEQ ID NO:8 and a carboxy-terminal cysteine-rich domain from about amino acids 182-263 of SEQ ID NO:8. The human Dkk-1 protein is a secreted protein which further contains a signal sequence at about amino acids 1-18, 1-19, 1-20, or 1-21 of SEQ ID NO:8. Accordingly, a mature human Dkk-1 protein begins at about

amino acid residue 19, 20, 21, or 22 of SEQ ID NO:8 and is about 248, 247, 246, or 245 amino acids in length.

The human Dkk-2 cDNA, which is approximately 3687 nucleotides in length, encodes a protein which is approximately 259 amino acid residues in length. The
5 human Dkk-2 protein contains a cysteine-rich region comprising two cysteine-rich domains. A Dkk cysteine-rich region can be found at least, for example, from about amino acids 78-256 of SEQ ID NO:21. The hDkk-2 cysteine-rich region comprises an amino-terminal cysteine-rich domain from about amino acids 78-127 of SEQ ID NO:21 and a carboxy-terminal cysteine-rich domain from about amino acids 176-256 of SEQ
10 ID NO:21. The human Dkk-2 protein is a secreted protein which further contains a signal sequence at about amino acids 1-31, 1-32, 1-33, or 1-34 of SEQ ID NO:21. Accordingly, a mature human Dkk-2 protein begins at about amino acid residue 32, 33, 34, or 35 of SEQ ID NO:21 and is about 228, 227, 226, or 225 amino acids in length.

Dkk proteins of the present invention can be used to identify additional Dkk-
15 related proteins or family members. For example, a protein having homology to hDkk-3 was identified using the nucleotide sequence encoding the N-terminal unique region of hDkk-3 to search a nucleotide sequence database. A human cDNA clone (Accession No.: AA397836) was identified from the dBEST database as having homology to hDkk-3 and was fully sequenced. The encoded protein is referred to herein as human "Soggy-
20 1" or "Dkk-like-N". The nucleotide and predicted amino acid sequence of human Soggy-1 are depicted in Figure 7. The nucleotide sequence of human Soggy-1 (SEQ ID NO:13) encodes a protein having 242 amino acids (SEQ ID NO:14). The nucleotide sequence of human Soggy-1 includes a 5' untranslated region containing nucleotides 1-74 of SEQ ID NO:13, a coding region containing nucleotides 75-800 of SEQ ID NO:13
25 (corresponding to nucleotides 1-726 of SEQ ID NO:15), and a 3' untranslated region containing nucleotides 801-928 of SEQ ID NO:13. The Soggy-1 protein (amino acid residues 32-132) has 25% identity to an N-terminal domain of human Dkk-3 (consisting of amino acid residues 22-140) as determined by ALIGN, Myers and Miller, (1989) *CABIOS*, utilizing a PAM120 weight residue table, a gap length penalty of 12, and a gap
30 penalty of 4.

Two murine cDNA clones were further identified from the database and fully sequenced. Combining the sequence information from these two clones resulted in a full-length sequence for murine Soggy-1. The nucleotide sequence and predicted amino acid sequence of murine Soggy-1 are depicted in Figure 8. The nucleotide sequence of
35 murine Soggy-1 (SEQ ID NO:26) encodes a protein having 230 amino acids (SEQ ID NO:27). The nucleotide sequence of murine Soggy-1 includes a 5' untranslated region containing nucleotides 1-56 of SEQ ID NO:26, a coding region containing nucleotides

57-746 of SEQ ID NO:26 (corresponding to SEQ ID NO:26), and a 3' untranslated region containing nucleotides 747-835 of SEQ ID NO:26. Human and murine Soggy-1 proteins display 59% overall identity. An alignment of human and murine Soggy proteins to human and murine Dkk-3 proteins is depicted in Figure 10.

5 In one embodiment, a Soggy protein is identified based on the presence of at least one soggy domain or "SGY" domain in the protein or corresponding nucleic acid molecule. As defined herein, a "SGY domain" includes a protein domain of a Soggy protein (*e.g.*, hSoggy-1) having an amino acid sequence of about 45-56 amino acids and having at least about 25-40% identity with amino acid residues 90-140 of hDkk-3
10 (leu90-glu140 of SEQ ID NO:2). In another embodiment, a "SGY domain" has 46-55, preferably 47-54, more preferably about 48-53, and even more preferably about 49-52 or 50-51 amino acids, and has at least about 27-38%, preferably about 28-37%, more preferably about 29-36%, even more preferably about 30-35%, and even more preferably about 31-34%, or 32-33% identity with amino acid residues 90-140 of hDkk-3 (Leu90-
15 Glu140 of SEQ ID NO:2). In yet another embodiment, a "SGY domain" has the following consensus sequence: L - P - X(3) - H - X - E - X(7) - G - N - X - T - X(3) - H - X(4) - K - X - T - X - N - X(2) - G - X(4) - S - E - X - V - X(2) - S - X(4) - E (SEQ ID NO:29). For example, human Soggy-1 has a SGY domain from about amino acid
20 acid residues 81-131 (Leu 81-Glu131 of SEQ ID NO:14) having 33% identity with amino acid residues 90-140 of hDkk-3 (leu90-glu140 of SEQ ID NO:2). Likewise, murine Soggy-1 has a SGY domain from about amino acid residues 71-120 (Leu 71-Glu120 of SEQ ID NO:27) having 33% identity with amino acid residues 90-140 of hDkk-3 (leu90-glu140 of SEQ ID NO:2). The SGY domains of human and murine Soggy-1 are depicted by shaded boxes in Figure 10.

25 In another embodiment of the invention, a Soggy protein has at least one SGY domain and a signal sequence. For example, in one embodiment, a hSoggy-1 protein contains a signal sequence of about amino acids 1-29, 1-30, 1-31, or 1-32 of SEQ ID NO:14. Accordingly, a mature hSoggy-1 protein begins at about amino acid residue 30, 31, 32, or 33 of SEQ ID NO:14 and is about 213, 212, 211, or 210 amino acids in length.
30 In another embodiment, a mSoggy-1 protein contains a signal sequence of about amino acids 1-19, 1-20, 1-21, or 1-22 of SEQ ID NO:27. Accordingly, a mature mSoggy-1 protein begins at about amino acid residue 211, 210, 209, or 208 of SEQ ID NO:28 and is about 213, 212, 211, or 210 amino acids in length.

35 Various aspects of the invention are described in further detail in the following subsections:

I. Isolated Nucleic Acid Molecules

One aspect of the invention pertains to isolated nucleic acid molecules that encode Dkk proteins or biologically active portions thereof, as well as nucleic acid fragments sufficient for use as hybridization probes to identify Dkk-encoding nucleic acids (*e.g.*, Dkk mRNA) and fragments for use as PCR primers for the amplification or mutation of Dkk nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated Dkk nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. An isolated chromosome is not an isolated nucleic acid molecule as defined herein. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or portion of the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, as a hybridization probe, Dkk nucleic acid molecules can be isolated using standard

hybridization and cloning techniques (*e.g.*, as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989*).

5 Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the
10 DNA insert of the plasmid deposited with ATCC as Accession Number _____, can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of
15 the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____.

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers
20 according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to Dkk nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In a preferred embodiment, an isolated nucleic acid molecule of the invention
25 comprises the nucleotide sequence shown in SEQ ID NO:1. The sequence of SEQ ID NO:1 corresponds to the human Dkk-3 cDNA. This cDNA comprises sequences encoding the human Dkk-3 protein (*i.e.*, "the coding region", from nucleotides 38-1087), as well as 5' untranslated sequences (nucleotides 1 to 37) and 3' untranslated sequences (nucleotides 1088 to 2479). Alternatively, the nucleic acid molecule can comprise only
30 the coding region of SEQ ID NO:1 (*e.g.*, nucleotides 38 to 1087, corresponding to SEQ ID NO:3). A plasmid containing the full-length nucleotide sequence encoding hDkk-3 was deposited with the American Type Culture Collection (ATCC), presently in Manassas Virginia, on June 11, 1997 and assigned Accession Number 98452.

In another preferred embodiment, an isolated nucleic acid molecule of the
35 invention comprises the nucleotide sequence shown in SEQ ID NO:4. The sequence of SEQ ID NO:4 corresponds to the human Dkk-4 cDNA. This cDNA comprises sequences encoding the human Dkk-4 protein (*i.e.*, "the coding region", from

nucleotides 125-796), as well as 5' untranslated sequences (nucleotides 1 to 124) and 3' untranslated sequences (nucleotides 797 to 848). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:4 (e.g., nucleotides 125 to 796, corresponding to SEQ ID NO:6).

5 In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:7. The sequence of SEQ ID NO:7 corresponds to the human Dkk-1 cDNA. This cDNA comprises sequences encoding the human Dkk-1 protein (*i.e.*, "the coding region", from nucleotides 109-906), as well as 5' untranslated sequences (nucleotides 1 to 108) and 3' untranslated sequences (nucleotides 907-1536). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:7 (e.g., nucleotides 109-906, corresponding to SEQ ID NO:9). A plasmid containing the full-length nucleotide sequence encoding hDkk-1 was deposited with the American Type Culture Collection (ATCC), presently in Manassas Virginia, on January 16, 1998 and assigned Accession
10
15 Number 98633.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:20. The sequence of SEQ ID NO:20 corresponds to the human Dkk-2 cDNA. This cDNA comprises sequences encoding the human Dkk-2 protein (*i.e.*, "the coding region", from nucleotides 724-1500), 5' untranslated sequences (nucleotides 1-723), as well as 3' untranslated sequences (nucleotides 1501-3687). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:20 (e.g., nucleotides 724 to 1500, corresponding to SEQ ID NO:22). A plasmid, clone fthu133, containing the full-length nucleotide sequence encoding hDkk-2 was deposited with the American
20
25 Type Culture Collection (ATCC), presently in Manassas Virginia, on March 2, 1999 and assigned Accession Number _____.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:13. The sequence of SEQ ID NO:13 corresponds to the human Soggy cDNA. This cDNA comprises sequences encoding the human Soggy protein (*i.e.*, "the coding region", from nucleotides 75 to 800), as well as 5' untranslated sequences (nucleotides 1 to 74) and 3' untranslated sequences (nucleotides 801 to 928). Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:13 (e.g., nucleotides 75 to 800, corresponding to SEQ ID NO:15).
30

35 In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or

SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number
5 _____, or a portion of any of these nucleotide sequences. A nucleic acid molecule which is complementary to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as
10. Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide
15 sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, such that it can hybridize to the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or
20 SEQ ID NO:20, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, thereby forming a stable duplex.

In still another preferred embodiment, an isolated nucleic acid molecule of the
25 present invention comprises a nucleotide sequence which is at least about 30-35%, preferably about 40-45%, more preferably about 50-55%, even more preferably about 60-65%, and even more preferably at least about 70-75%, 80-85%, 90-95% or more homologous to the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15,
30 SEQ ID NO:20, or SEQ ID NO:22, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, or a portion of any of these nucleotide sequences.

35 In one aspect, the present invention features isolated nucleic acid molecules which are linear (*e.g.*, linear fragments of double-stranded DNA, linear strands of single-stranded DNA, single-stranded RNA molecules, and oligonucleotides). Another aspect

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of the present invention features circular nucleic acid molecules (*e.g.*, double-stranded DNA molecules, for example, plasmid molecules including the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:20, or SEQ ID NO:22, the
5 nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, or a portion of any of these nucleotide sequences).

10 In one embodiment, the isolated nucleic acid molecules of the present invention are DNA molecules which are in a form suitable for expression (*e.g.*, suitable for expression of corresponding messenger RNA or mRNA). In another embodiment, the isolated nucleic acid molecules are DNA molecules which are in a form suitable for
15 expression of corresponding protein (*e.g.*, in a form, for example, in a vector, which is capable of expressing protein, *e.g.*, in the appropriate orientation for expression from regulatory elements and/or in-frame with appropriate regulatory elements). In another embodiment, the isolated nucleic acids are in a form suitable for determination of nucleic acid sequence (*e.g.*, in a form suitable for sequencing, for example, is a
20 sequencing vector including a M13, T7, T3 and SP6 promoter. Examples of sequencing vectors include, but are not limited to pBluescript (Stratagene™), pT7T3D (Pharmacia™) and pCR2.1 (InVitrogen). In yet another embodiment, the isolated nucleic acid molecules are free from vector sequences. In a preferred embodiment, an isolated nucleic acid molecule is free from sequencing vector sequences.

Moreover, the nucleic acid molecule of the invention can comprise only a portion
25 of the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as
30 Accession Number _____, for example a fragment which can be used as a probe or primer or a fragment encoding a biologically active portion of a Dkk protein or Dkk-related protein. The nucleotide sequence determined from the cloning of the human Dkk genes allows for the generation of probes and primers designed for use in identifying and/or cloning Dkk homologues in other cell types, *e.g.*, from other tissues, as well as
35 Dkk homologues from other mammals and Dkk-related proteins. The probe/primer typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to

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at least about 12, preferably about 25, more preferably about 40, 50 or 75 consecutive nucleotides of a sense sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, of an anti-sense sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, or of a naturally occurring mutant of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____. In an exemplary embodiment, a nucleic acid molecule of the present invention comprises a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule consisting of nucleotides 470-2479 of SEQ ID NO:1 or to a nucleic acid molecule consisting of nucleotides 1-475 of SEQ ID NO:4.

Probes based on human nucleotide sequences (*e.g.*, the human Dkk nucleotide sequence) can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. For instance, primers based on the nucleic acid represented in SEQ ID NOs:1 or 3 can be used in PCR reactions to clone Dkk homologs (*e.g.*, hDkk-3 homologues). In a preferred embodiment of the invention, Dkk homologs are cloned by PCR amplification (*e.g.*, RT-PCR) using primers hybridizing to a portion of the nucleotide sequence encoding the Dkk cysteine rich domain. Likewise, probes based on the subject Dkk sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto, *e.g.*, the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a Dkk protein, such as by measuring a level of a Dkk-encoding nucleic acid in a sample of cells from a subject *e.g.*, detecting Dkk mRNA levels or determining whether a genomic Dkk gene has been mutated or deleted.

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A nucleic acid fragment encoding a "biologically active portion of a Dkk or Dkk-related protein" can be prepared by isolating a portion of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, which encodes a polypeptide having a biological activity (the biological activities of the Dkk and Dkk-related proteins have previously been described), expressing the encoded portion of the protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the protein.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, due to degeneracy of the genetic code and thus encode the same proteins as those encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____. In another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO:2, SEQ ID NO: 5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21.

In addition to the human nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of the Dkk or Dkk-related proteins may exist within a population (*e.g.*, the human population). Such genetic polymorphism in the Dkk or Dkk-related genes may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules

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comprising an open reading frame encoding a protein, preferably a mammalian Dkk or Dkk-related protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of a Dkk or Dkk-related gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in genes that are the result of natural allelic variation and that do not alter the functional activity of a Dkk or Dkk-related protein are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding Dkk or Dkk-related proteins from other species, and thus which have a nucleotide sequence which differs from the human sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, are intended to be within the scope of the invention. For example, a murine Dkk-3 cDNA has been identified based of the nucleotide sequence of human Dkk-3. The nucleotide sequence of murine Dkk-3 (SEQ ID NO:16) encodes a hDkk-3 protein having 349 amino acids. The nucleotide and amino acid sequences of murine Dkk-3 are depicted in Figure 5. The coding region of murine Dkk-3 is represented by SEQ ID NO:18. A plasmid containing the full-length nucleotide sequence encoding mDkk-3 was deposited with the American Type Culture Collection (ATCC), presently in Manassas, Virginia, on January 16, 1998 and assigned Accession Number 98634. Likewise, a murine Dkk-related protein (Soggy-1) has been identified based of the nucleotide sequence of human Dkk-3. The nucleotide sequence of murine Soggy-1 (SEQ ID NO:26) encodes a protein having 230 amino acids (SEQ ID NO:27). The nucleotide and amino acid sequences of murine Soggy-1 are depicted in Figure 8. The coding region of murine Soggy-1 is represented by SEQ ID NO:28.

Nucleic acid molecules corresponding to natural allelic variants and homologues of the Dkk or Dkk-related cDNAs of the invention can be isolated based on their homology to the human nucleic acids disclosed herein using the human cDNA, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. Examples of tissues and/or libraries suitable for isolation of the subject nucleic acids include brain, spinal chord and heart tissue. cDNA encoding a Dkk protein (*e.g.*, a hDkk-3 protein) can be obtained by isolating total mRNA from a cell, *e.g.*, a vertebrate cell, a mammalian cell, or a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a hDkk-3 protein can also be

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cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. The nucleic acid of the invention can be DNA or RNA or analogs thereof.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 15 nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____. In another embodiment, the nucleic acid is at least 30, 50, 100, 250, 300, 400 or 500 nucleotides in length. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:1 corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

In addition to naturally-occurring allelic variants of the Dkk or Dkk-related sequences that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, thereby leading to changes in the amino acid sequence of the encoded Dkk proteins, without altering the functional ability of the Dkk proteins. For example, nucleotide substitutions leading to amino acid

substitutions (particularly conservative amino acid substitutions) at "non-essential" amino acid residues can be made in the sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of Dkk (or wild-type Dkk-related sequence) (*e.g.*, the sequence of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21) without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the Dkk or Dkk-related proteins of the present invention (*e.g.*, cysteine residues within cysteine-rich domains), are predicted to be particularly unamenable to alteration. Furthermore, amino acid residues that are conserved between Dkk protein and other proteins having cysteine-rich domains are not likely to be amenable to alteration.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding Dkk or Dkk-related proteins that contain changes in amino acid residues that are not essential for activity. Such proteins differ in amino acid sequence from SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21 yet retain biological activity. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21. Preferably, the protein encoded by the nucleic acid molecule is at least about 65-70% homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, more preferably at least about 75-80% homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, even more preferably at least about 85-90% homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, and most preferably at least about 95% homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21.

An isolated nucleic acid molecule encoding a Dkk protein homologous to the protein of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of

"SEQ ID NO:2"

the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into

5 SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, by

10 standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been

15 defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*,

20 threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, for example, a predicted nonessential amino acid residue in a Dkk protein (*e.g.*, one not located in a cysteine-rich domain) is preferably replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a Dkk or

25 Dkk-related coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of

30 the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number _____, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

In a preferred embodiment, a mutant Dkk or Dkk-related protein can be assayed

35 for intracellular calcium, an increase in phosphatidylinositol or other molecule, and can result, *e.g.*, in phosphorylation of specific proteins, a modulation of gene transcription and any of the other biological activities set forth herein.

In a preferred embodiment, a mutant Dkk or Dkk-related protein can also be assayed for the ability to (1) modulate cellular signal transduction, either *in vitro* or *in vivo*; (2) regulate communication between cells; (3) regulate expression of genes whose expression is modulated by binding of Dkk (*e.g.*, hDkk-3) to a receptor; (4) regulate gene transcription in a cell involved in development or differentiation, either *in vitro* or *in vivo*; (5) regulate cellular proliferation, either *in vitro* or *in vivo*; (6) form and/or maintain ordered spatial arrangements of differentiated tissues in vertebrates; (7) modulate cell death (*e.g.* cell survival); (8) regulate cell migration; and/or (9) modulate immune system function.

In addition to the nucleic acid molecules encoding Dkk or Dkk-related proteins described above, another aspect of the invention pertains to isolated nucleic acid molecules which are antisense thereto. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire Dkk coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding Dkk. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues (*e.g.*, the coding region of human Dkk-3 corresponds to SEQ ID NO:3, the coding region of human Dkk-4 corresponds to SEQ ID NO:6, the coding region of human Dkk-1 corresponds to SEQ ID NO:9, the coding region of human Dkk-2 corresponds to SEQ ID NO:22, and the coding region of human Soggy corresponds to SEQ ID NO:15). In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding a Dkk or Dkk-related protein. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

Given the coding strand sequences disclosed herein (*e.g.*, SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:15, or SEQ ID NO:22), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of a Dkk or Dkk-related mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of the mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of Dkk mRNA. An antisense oligonucleotide can be, for example,

about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90 nucleotides in length. In a preferred embodiment, an oligonucleotide is about 30-90, preferably about 40-80, more preferably about 50-70 nucleotides in length and is antisense to a portion of SEQ ID NO:1 from about nucleotides 1-150. In another embodiment, an

5 oligonucleotide is antisense to a portion of SEQ ID NO:4 from about nucleotides 25-225. In another embodiment, an oligonucleotide is antisense to a portion of SEQ ID NO:7 from about nucleotides 1-200. In another embodiment, an oligonucleotide is antisense to a portion of SEQ ID NO:20 from about nucleotides 625-825. In yet another embodiment, an oligonucleotide is antisense to a portion of SEQ ID NO:13 from about
10 nucleotides 1-175.

An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides
15 designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives, acridine substituted nucleotides, can be used. Alternatively, the antisense nucleic acid molecule can be synthesized to increase transport across cellular membranes, *e.g.*, methylphosphonate derivatives. The antisense
20 molecules can include a 3'-terminal cap (*e.g.*, a 3'-aminopropyl modification), a biotin moiety, or even a 3'-3' terminal linkage.

Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-
25 carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-
30 mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the
35 antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA

transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA
5 and/or genomic DNA encoding a Dkk or Dkk-related protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix.
10 An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*,
15 by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter
20 are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier *et al.*, (1987) *Nucleic Acids Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-*o*-methylribonucleotide (Inoue *et al.*, (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.*, (1987) *FEBS Lett.* 215:327-330).
25

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are
30 capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave Dkk or Dkk-related mRNA transcripts to thereby inhibit translation of Dkk or Dkk-related mRNA. A ribozyme having specificity for a Dkk- or Dkk-
35 related-encoding nucleic acid can be designed based upon the nucleotide sequence of a Dkk or Dkk-related cDNA disclosed herein (*i.e.*, SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the nucleotide sequence of the DNA insert of the

plasmid deposited with ATCC as Accession Number 98452, the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 98633). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be
5 cleaved in a Dkk-encoding mRNA. See, *e.g.*, Cech *et al.*, U.S. Patent No. 4,987,071; and Cech *et al.*, U.S. Patent No. 5,116,742. Alternatively, Dkk (or Dkk-related) mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

10 Alternatively, gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the Dkk or Dkk-related gene (*e.g.*, the promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. *et al.*, (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J.
15 (1992) *Bioassays* 14(12):807-15.

In yet another embodiment, the nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to
20 generate peptide nucleic acids (see Hyrup B. *et al.* (1996) *Bioorganic & Medicinal Chemistry* 4 (1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific
25 hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup B. *et al.* (1996) *supra*; Perry-O'Keefe *et al.* PNAS 93: 14670-675.

PNAs of Dkk or Dkk-related nucleic acid molecules can be used therapeutic and
30 diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of Dkk or Dkk-related nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as 'artificial restriction enzymes' when
35 used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup B. (1996) *supra*)); or as probes or primers for DNA sequencing or hybridization (Hyrup B. *et al.* (1996) *supra*; Perry-O'Keefe *supra*).

In another embodiment, PNAs of Dkk can be modified, (*e.g.*, to enhance their stability or cellular uptake), by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of Dkk nucleic acid molecules can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, (*e.g.*, RNase H and DNA polymerases), to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup B. (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup B. (1996) *supra* and Finn P.J. *et al.* (1996) *Nucleic Acids Res.* 24 (17): 3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, *e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag, M. *et al.* (1989) *Nucleic Acid Res.* 17: 5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn P.J. *et al.* (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K.H. *et al.* (1975) *Bioorganic Med. Chem. Lett.* 5: 1119-11124).

In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. US.* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134, published April 25, 1988). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.* (1988) *BioTechniques* 6:958-976) or intercalating agents. (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

II. Isolated Dkk Proteins and Anti-Dkk Antibodies

One aspect of the invention pertains to isolated Dkk proteins, Dkk-related proteins and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise antibodies. In one embodiment, native Dkk or Dkk-related proteins can be isolated from cells or tissue sources by an appropriate

purification scheme using standard protein purification techniques. In another embodiment, proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a Dkk or Dkk-related protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

5 An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the Dkk or Dkk-related protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of protein in
10 which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of Dkk or Dkk-related protein having less than about 30% (by dry weight) of non-Dkk protein or non-Dkk-related protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of
15 non-Dkk protein or non-Dkk-related protein, still more preferably less than about 10% of non-Dkk protein or non-Dkk-related protein, and most preferably less than about 5% non-Dkk protein or non-Dkk-related protein. When the Dkk or Dkk-related protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about
20 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of Dkk or Dkk-related protein in which the protein is separated
25 from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of Dkk protein having less than about 30% (by dry weight) of chemical precursors, non-Dkk chemicals, or non-Dkk-related chemicals, more preferably less than about 20% chemical precursors, non-Dkk chemicals, or non-Dkk-related chemicals, still more preferably less than about 10% chemical precursors,
30 non-Dkk chemicals, or non-Dkk-related chemicals, and most preferably less than about 5% chemical precursors, non-Dkk chemicals, or non-Dkk-related chemicals.

Biologically active portions of a Dkk or Dkk-related protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the amino acid sequence of the Dkk or Dkk-related protein, *e.g.*, the amino acid sequence shown in
35 SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, which include less amino acids than the full length proteins, and exhibit at least one activity of a Dkk or Dkk-related protein. Typically, biologically active portions comprise a domain

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or motif with at least one activity of the Dkk or Dkk-related protein. A biologically active portion of a protein can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length.

In one embodiment, a biologically active portion of a Dkk protein comprises at least a cysteine-rich region. In another embodiment, a biologically active portion of a Dkk protein comprises at least a cysteine-rich region, wherein the cysteine-rich region includes at least one cysteine-rich domain. In yet another embodiment, a biologically active portion of a Dkk protein comprises at least a signal sequence.

In another embodiment, a biologically active portion of a Dkk-related protein (*e.g.*, a Soggy protein) comprises at least a Soggy domain. In yet another embodiment, a biologically active portion of a Dkk-related protein comprises at least a signal sequence.

In an alternative embodiment, a biologically active portion of a Dkk or Dkk-related protein comprises an amino acid sequence lacking a signal sequence.

It is to be understood that a preferred biologically active portion of a Dkk or Dkk-related protein of the present invention may contain at least one of the above-identified structural domains. A more preferred biologically active portion of a Dkk or Dkk-related protein may contain at least two of the above-identified structural domains. An even more preferred biologically active portion of a protein may contain at least three of the above-identified structural domains. A particularly preferred biologically active portion of a protein of the present invention may contain at least four of the above-identified structural domains.

Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native Dkk or Dkk-related protein.

In a preferred embodiment, the Dkk protein has an amino acid sequence shown in SEQ ID NO:2 or an amino acid sequence at least about 55% homologous to SEQ ID NO:2. In another preferred embodiment, the Dkk protein has an amino acid sequence shown in SEQ ID NO:5 or an amino acid sequence at least about 35% homologous to SEQ ID NO:5. In another preferred embodiment, the Dkk protein has an amino acid sequence shown in SEQ ID NO:8 or an amino acid sequence at least about 85% homologous to SEQ ID NO:8. In another preferred embodiment, the Dkk protein has an amino acid sequence shown in SEQ ID NO:21 or an amino acid sequence at least about 35% homologous to SEQ ID NO:21. In another preferred embodiment, the protein has an amino acid sequence shown in SEQ ID NO:14 or an amino acid sequence at least about 60% homologous to SEQ ID NO:14. In still another preferred embodiment, a protein of the present invention comprises an amino acid sequence which is at least about 30-35%, preferably about 40-45%, more preferably about 50-55%, even more

preferably about 60-65%, and even more preferably at least about 70-75%, 80-85%, 90-95% or more homologous to the amino acid sequences shown in SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21.

In other embodiments, the protein is substantially homologous to SEQ ID NO:2, 5 SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, and, preferably, retains the functional activity of the protein of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail in subsection I above. Accordingly, in another embodiment, the protein is a protein which comprises an amino 10 acid sequence at least about 60% homologous to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21 and, preferably, retains the functional activity of the proteins of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:11, respectively. Preferably, the protein is at least about 70% homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID 15 NO:14, or SEQ ID NO:21, more preferably at least about 80% homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, even more preferably at least about 90% homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21, and most preferably at least about 95% or more homologous to SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or 20 SEQ ID NO:21.

To determine the percent homology of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence and non-homologous sequences 25 can be disregarded for comparison purposes). In one embodiment, an alignment is a global alignment, *e.g.*, an overall sequence alignment. In another embodiment, an alignment is a local alignment. In a preferred embodiment, the length of a sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at 30 least 70%, 80%, or 90% of the length of the reference sequence to which it is aligned (*e.g.*, when aligning a second sequence to the Dkk amino acid sequence of SEQ ID NO:2, at least 105, preferably at least 145, more preferably at least 175, even more preferably at least 210, and even more preferably at least 245, 280 or 315 amino acid residues are aligned). The amino acid residues or nucleotides at corresponding amino 35 acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used

herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of
5 the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated
10 into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, 5, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a
15 NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (*CABIOS*, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a
20 gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J.*
25 *Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to Dkk nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to Dkk protein molecules of the invention. To obtain
30 gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

The invention also provides Dkk or Dkk-related chimeric or fusion proteins. As
35 used herein, a "chimeric protein" or "fusion protein" comprises a Dkk or Dkk-related polypeptide operatively linked to a non-Dkk polypeptide or non-Dkk-related polypeptide. A "Dkk polypeptide" or "Dkk-related polypeptide" refers to a polypeptide

having an amino acid sequence corresponding to Dkk or a Dkk-related protein, whereas a "non-Dkk polypeptide" or "non-Dkk-related polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the Dkk or Dkk-related protein, *e.g.*, a protein which is different from the Dkk or Dkk-related protein and which is derived from the same or a different organism. Within a Dkk or Dkk-related fusion protein the Dkk or Dkk-related polypeptide can correspond to all or a portion of a Dkk or Dkk-related protein. In a preferred embodiment, a Dkk or Dkk-related fusion protein comprises at least one biologically active portion of a Dkk protein. In another preferred embodiment, a Dkk or Dkk-related fusion protein comprises at least two biologically active portions of a Dkk or Dkk-related protein. In another preferred embodiment, a Dkk or Dkk-related fusion protein comprises at least three biologically active portions of a Dkk or Dkk-related protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the Dkk or Dkk-related polypeptide and the non-Dkk or non-Dkk-related polypeptide are fused in-frame to each other. The non-Dkk or non-Dkk-related polypeptide can be fused to the N-terminus or C-terminus of the Dkk or Dkk-related polypeptide.

For example, in one embodiment, the fusion protein is a GST-Dkk fusion protein in which the Dkk sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant Dkk.

In another embodiment, the fusion protein is a Dkk or Dkk-related protein containing a heterologous signal sequence at its N-terminus. For example, the native Dkk signal sequence (*i.e.*, about amino acids 1 to 23 of SEQ ID NO:2) can be removed and replaced with a signal sequence from another protein. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of Dkk or Dkk-related proteins can be increased through use of a heterologous signal sequence.

In yet another embodiment, the fusion protein is a Dkk-immunoglobulin fusion protein in which the Dkk sequences comprising primarily the Dkk cysteine-rich regions are fused to sequences derived from a member of the immunoglobulin protein family. Soluble derivatives have also been made of cell surface glycoproteins in the immunoglobulin gene superfamily consisting of an extracellular domain of the cell surface glycoprotein fused to an immunoglobulin constant (Fc) region (see *e.g.*, Capon, *et al.* (1989) *Nature* 337:525-531 and Capon U.S. Patents 5,116,964 and 5,428,130 [CD4-IgG1 constructs]; Linsley, P.S. *et al.* (1991) *J. Exp. Med.* 173:721-730 [a CD28-IgG1 construct and a B7-1-IgG1 construct]; and Linsley, P.S. *et al.* (1991) *J. Exp. Med.* 174:561-569 and U.S. Patent 5,434,131 [a CTLA4-IgG1]). Such fusion proteins have proven useful for modulating receptor-ligand interactions. Soluble derivatives of cell surface proteins of the tumor necrosis factor receptor (TNFR) superfamily proteins have

been made consisting of an extracellular domain of the cell surface receptor fused to an immunoglobulin constant (Fc) region (See for example Moreland *et al.* (1997) N. Engl. J. Med. 337(3):141-147; van der Poll *et al.* (1997) Blood 89(10):3727-3734; and Ammann *et al.* (1997) J. Clin. Invest. 99(7):1699-1703.)

5 The Dkk-immunoglobulin fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject to inhibit an interaction between a Dkk ligand and a Dkk receptor on the surface of a cell, to thereby suppress Dkk-mediated signal transduction *in vivo*. The Dkk-immunoglobulin fusion proteins can be used to affect the bioavailability of a Dkk cognate receptor. Inhibition of the Dkk
10 ligand/Dkk interaction may be useful therapeutically for both the treatment of differentiative or proliferative disorders, as well as modulating (*e.g.*, promoting or inhibiting) developmental responses, cell adhesion, and/or cell fate. Moreover, the Dkk-immunoglobulin fusion proteins of the invention can be used as immunogens to produce anti-Dkk antibodies in a subject, to purify Dkk ligands and in screening assays to
15 identify molecules which inhibit the interaction of Dkk with a Dkk ligand.

 Preferably, a Dkk or Dkk-related chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or
20 stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried
25 out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST
30 polypeptide). A Dkk-encoding nucleic acid or nucleic acid encoding a Dkk-related protein can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the protein.

 The present invention also pertains to variants of the Dkk or Dkk-related proteins which function as either agonists (mimetics) or as antagonists. Variants of the
35 Dkk or Dkk-related proteins can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of a Dkk or Dkk-related protein. An agonist of the Dkk or Dkk-related proteins can retain substantially the same, or a subset, of the biological activities

of the naturally occurring form of a Dkk or Dkk-related protein. An antagonist of a Dkk or Dkk-related protein can inhibit one or more of the activities of the naturally occurring form of the protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cascade which includes the Dkk or Dkk-related protein.

5 Thus, specific biological effects can be elicited by treatment with a variant of limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the Dkk or Dkk-related protein.

10 In one embodiment, variants of a Dkk or Dkk-related protein which function as either agonists (mimetics) or as antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of a Dkk or Dkk-related protein for protein agonist or antagonist activity. In one embodiment, a variegated library of Dkk or Dkk-related variants is generated by combinatorial mutagenesis at the nucleic acid level and
15 is encoded by a variegated gene library. A variegated library of Dkk or Dkk-related variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential Dkk or Dkk-related sequences is expressible as individual polypeptides, or alternatively, as a set of
20 larger fusion proteins (*e.g.*, for phage display) containing the set of Dkk or Dkk-related sequences therein. There are a variety of methods which can be used to produce libraries of potential Dkk or Dkk-related variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate
25 expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential Dkk or Dkk-related sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura *et al.*, (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.*, (1984) *Science* 198:1056; Ike *et al.*, (1983) *Nucleic Acid Res.* 11:477.

30 In addition, libraries of fragments of a Dkk or Dkk-related protein coding sequence can be used to generate a variegated population of Dkk or Dkk-related fragments for screening and subsequent selection of variants of a Dkk or Dkk-related protein. In one embodiment, a library of coding sequence fragments can be generated by
35 treating a double stranded PCR fragment of a Dkk coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded

portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the Dkk protein.

5 Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of Dkk or Dkk-related proteins. The most widely used techniques, which are amenable to high
10 through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique
15 which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify Dkk variants (Arkin and Yourvan (1992) *PNAS* 89:7811-7815; Delgrave *et al.*, (1993) *Protein Engineering* 6(3):327-331).

 In one embodiment, cell based assays can be exploited to analyze a variegated Dkk or Dkk-related library. For example, a library of expression vectors can be
20 transfected into a cell line which ordinarily responds to a particular ligand in a Dkk-dependent manner. The transfected cells are then contacted with the ligand and the effect of expression of the mutant on signaling by the ligand can be detected, *e.g.*, by measuring any of a number of immune cell responses. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of
25 ligand induction, and the individual clones further characterized.

 An isolated Dkk protein, Dkk-related protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind Dkk or Dkk-related proteins using standard techniques for polyclonal and monoclonal antibody preparation. A full-length Dkk or Dkk-related protein can be used or, alternatively, the invention
30 provides antigenic peptide fragments for use as immunogens. The antigenic peptide of Dkk comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:8, SEQ ID NO:14, or SEQ ID NO:21 and encompasses an epitope of Dkk or Dkk-related protein such that an antibody raised
35 against the peptide forms a specific immune complex with the protein. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

Preferred epitopes encompassed by the antigenic peptide are regions of Dkk or Dkk-related proteins that are located on the surface of the protein, *e.g.*, hydrophilic regions.

5 A Dkk or Dkk-related immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed Dkk or Dkk-related protein or a chemically synthesized Dkk or Dkk-related polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent.

10 Immunization of a suitable subject with an immunogenic Dkk preparation, for example, induces a polyclonal anti-Dkk antibody response.

Accordingly, another aspect of the invention pertains to anti-Dkk antibodies as well as antibodies to Dkk-related proteins. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin

15 molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as Dkk or Dkk-related antigens. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')₂ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind Dkk

20 or Dkk-related polypeptides. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of Dkk or a or Dkk-related protein. A monoclonal antibody composition thus typically displays a single binding affinity for a particular Dkk or Dkk-related protein

25 with which it immunoreacts.

Polyclonal antibodies can be prepared as described above by immunizing a suitable subject with a Dkk or Dkk-related immunogen. The antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized Dkk or Dkk-related

30 protein. If desired, the antibody molecules directed against Dkk or Dkk-related protein can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal

35 antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown *et al.*, (1981) *J. Immunol.* 127:539-46; Brown *et al.*, (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.*,

(1976) *PNAS* 76:2927-31; and Yeh *et al.*, (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.*, (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole *et al.*, (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for
5 producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.*, (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an
10 immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a Dkk or Dkk-related immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds Dkk or Dkk-related protein.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating a monoclonal
15 antibody (see, *e.g.*, G. Galfré *et al.*, (1977) *Nature* 266:55052; Gefter *et al.*, *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian
20 species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines
25 can be used as a fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively
30 fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind Dkk or Dkk-related protein, *e.g.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a
35 monoclonal antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with Dkk or Dkk-related protein to thereby isolate immunoglobulin library members that bind

Dkk or Dkk-related protein. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use

5 in generating and screening antibody display library can be found in, for example, Ladner *et al.*, U.S. Patent No. 5,223,409; Kang *et al.*, PCT International Publication No. WO 92/18619; Dower *et al.*, PCT International Publication No. WO 91/17271; Winter *et al.*, PCT International Publication WO 92/20791; Markland *et al.*, PCT International Publication No. WO 92/15679; Breitling *et al.*, PCT International Publication WO

10 93/01288; McCafferty *et al.*, PCT International Publication No. WO 92/01047; Garrard *et al.*, PCT International Publication No. WO 92/09690; Ladner *et al.*, PCT International Publication No. WO 90/02809; Fuchs *et al.*, (1991) *Bio/Technology* 9:1370-1372; Hay *et al.*, (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.*, (1989) *Science* 246:1275-1281; Griffiths *et al.*, (1993) *EMBO J* 12:725-734; Hawkins *et al.*, (1992) *J. Mol. Biol.*

15 226:889-896; Clarkson *et al.*, (1991) *Nature* 352:624-628; Gram *et al.*, (1992) *PNAS* 89:3576-3580; Garrard *et al.*, (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.*, (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas *et al.*, (1991) *PNAS* 88:7978-7982; and McCafferty *et al.*, *Nature* (1990) 348:552-554.

Additionally, recombinant antibodies, such as chimeric and humanized

20 monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.*, International Application No. PCT/US86/02269; Akira, *et al.*, European

25 Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.*, European Patent Application 173,494; Neuberger *et al.*, PCT International Publication No. WO 86/01533; Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent Application 125,023; Better *et al.*, (1988) *Science* 240:1041-1043; Liu *et al.*, (1987) *PNAS* 84:3439-3443; Liu *et al.*, (1987) *J. Immunol.*

30 139:3521-3526; Sun *et al.*, (1987) *PNAS* 84:214-218; Nishimura *et al.*, (1987) *Canc. Res.* 47:999-1005; Wood *et al.*, (1985) *Nature* 314:446-449; and Shaw *et al.*, (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.*, (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.*, (1986) *Nature* 321:552-525; Verhoeyan *et al.*, (1988) *Science* 239:1534; and Beidler *et al.*,

35 (1988) *J. Immunol.* 141:4053-4060.

An antibody (e.g., monoclonal antibody) can be used to isolate Dkk or Dkk-related protein by standard techniques, such as affinity chromatography or

immunoprecipitation. An antibody can facilitate the purification of natural Dkk or Dkk-related protein from cells and of recombinantly produced Dkk or Dkk-related protein expressed in host cells. Moreover, an antibody can be used to detect Dkk or Dkk-related protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the Dkk or Dkk-related protein. Antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

20 III. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding Dkk or a nucleic acid encoding a Dkk-related protein (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include

such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

5 The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of
10 interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for
15 example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art
20 that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, Dkk proteins, Dkk-related proteins, mutant forms of Dkk or Dkk-
25 related proteins, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of Dkk or Dkk-related proteins in prokaryotic or eukaryotic cells. For example, Dkk can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are
30 discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

35 Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion

vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Purified fusion proteins can be utilized in activity assays, in ligand binding (*e.g.*, direct assays or competitive assays described in detail below), to generate antibodies specific for Dkk or Dkk-related proteins, as examples. In a preferred embodiment, a Dkk or Dkk-related fusion expressed in a retroviral expression vector of the present invention can be utilized to infect bone marrow cells which are subsequently transplanted into irradiated recipients. The pathology of the subject recipient is then examined after sufficient time has passed (*e.g.* six (6) weeks).

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid *trp-lac* fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 *gn10-lac* fusion promoter mediated by a coexpressed viral RNA polymerase (T7 *gn1*). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 *gn1* gene under the transcriptional control of the *lacUV 5* promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the Dkk or Dkk-related expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.*, (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), pYES2
5 (Invitrogen Corporation, San Diego, CA), and picZ (Invitrogen Corp, San Diego, CA).

Alternatively, Dkk or Dkk-related protein can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology*
10 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.*, (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the
15 expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed.*, Cold Spring
20 Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.*, (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.*, (1983) *Cell*
30 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund *et al.*, (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters
35 are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to Dkk mRNA or a Dkk-related mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, Dkk protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.*, (*Molecular Cloning: A Laboratory Manual. 2nd, ed.*, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding Dkk or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a Dkk or Dkk-related protein. Accordingly, the invention further provides methods for producing Dkk or Dkk-related proteins using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding Dkk or a Dkk-related protein has been introduced) in a suitable medium such that protein is produced. In another embodiment, the method further comprises isolating Dkk or a Dkk-related protein from the medium or the host cell.

The host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which Dkk-coding sequences (or Dkk-related coding sequences) have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous Dkk sequences (or Dkk-related sequences) have been introduced into their genome or homologous recombinant animals in which endogenous Dkk sequences (or Dkk-related sequences) have been altered. Such animals are useful for studying the function and/or activity of Dkk or Dkk-related proteins and for identifying and/or evaluating modulators of Dkk or Dkk-related protein activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous

Dkk or Dkk-related gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created, for example, by introducing
5 Dkk-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by
microinjection, retroviral infection, and allowing the oocyte to develop in a
pseudopregnant female foster animal. The human Dkk cDNA sequence of SEQ ID
NO:1, SEQ ID NO:4, SEQ ID NO:7, or SEQ ID NO:20 can be introduced as a transgene
into the genome of a non-human animal. Alternatively, a nonhuman homologue of a
10 human Dkk gene, such as a mouse Dkk gene, can be isolated based on hybridization to
the human Dkk cDNA (described further in subsection I above) and used as a transgene.
Intronic sequences and polyadenylation signals can also be included in the transgene to
increase the efficiency of expression of the transgene. A tissue-specific regulatory
sequence(s) can be operably linked to the Dkk transgene to direct expression of Dkk
15 protein to particular cells. Methods for generating transgenic animals via embryo
manipulation and microinjection, particularly animals such as mice, have become
conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and
4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.*, and in
Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press,
20 Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other
transgenic animals. A transgenic founder animal can be identified based upon the
presence of the Dkk transgene in its genome and/or expression of Dkk mRNA in tissues
or cells of the animals. A transgenic founder animal can then be used to breed additional
animals carrying the transgene. Moreover, transgenic animals carrying a transgene
25 encoding Dkk can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains
at least a portion of a Dkk gene into which a deletion, addition or substitution has been
introduced to thereby alter, *e.g.*, functionally disrupt, the Dkk gene. The Dkk gene can
be a human gene (*e.g.*, the cDNA of SEQ ID NO:3, SEQ ID NO: 6, SEQ ID NO:9 or
30 SEQ ID NO:22), but more preferably, is a non-human homologue of a human Dkk gene.
For example, a mouse Dkk gene of SEQ ID NO:16 can be used to construct a
homologous recombination vector suitable for altering an endogenous Dkk gene in the
mouse genome. In a preferred embodiment, the vector is designed such that, upon
homologous recombination, the endogenous Dkk gene is functionally disrupted (*i.e.*, no
35 longer encodes a functional protein; also referred to as a "knock out" vector).
Alternatively, the vector can be designed such that, upon homologous recombination,
the endogenous Dkk gene is mutated or otherwise altered but still encodes functional

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protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous Dkk protein). In the homologous recombination vector, the altered portion of the Dkk gene is flanked at its 5' and 3' ends by additional nucleic acid of the Dkk gene to allow for homologous recombination to occur between the

5 exogenous Dkk gene carried by the vector and an endogenous Dkk gene in an embryonic stem cell. The additional flanking Dkk nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous

10 recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced Dkk gene has homologously recombined with the endogenous Dkk gene are selected (see *e.g.*, Li, E. *et al.*, (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and*

15 *Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline

20 transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.* It is also within the

25 scope of the present invention to practice the above-described transgenic methodology utilizing nucleic acid molecules which encode Dkk-related proteins.

In another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For

30 a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.*, (1992) *PNAS* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.*, (1991) *Science* 251:1351-1355. If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are

35 required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. *et al.*, (1997) *Nature* 385:810-813. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G₀ phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

IV. Pharmaceutical Compositions

The Dkk and Dkk-related nucleic acid molecules, Dkk and Dkk-related proteins, and anti-Dkk or anti-Dkk-related protein antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be

applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as

5 microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

10 For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be

15 permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in

20 the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will

25 protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

30 The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

35 It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject

to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.*, (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

V. Uses and Methods of the Invention

5 The molecules of the present invention (*e.g.*, nucleic acid molecules, proteins, protein homologues, and antibodies described herein) can be used in one or more of the following methods: a) screening assays; b) predictive medicine (*e.g.*, diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenetics); and c) methods of treatment (*e.g.*, therapeutic and prophylactic). As described herein, a Dkk protein of the
10 invention has one or more of the following activities: intracellular calcium, an increase in phosphatidylinositol or other molecule, and can result, *e.g.*, in phosphorylation of specific proteins, a modulation of gene transcription and any of the other biological activities set forth herein.

In a preferred embodiment, a Dkk activity is at least one or more of the following
15 activities: (i) interaction of a Dkk protein with and/or binding to a second molecule, (*e.g.*, a protein, such as a Dkk (*e.g.*, hDkk-3) receptor, a soluble form of a Dkk receptor, a receptor for a member of the *wnt* family of signaling proteins, or a non-Dkk signaling molecule); (ii) interaction of a Dkk protein with an intracellular protein via a membrane-bound Dkk receptor; (iii) complex formation between a soluble Dkk protein and a
20 second soluble Dkk binding partner (*e.g.*, a non-Dkk protein molecule or a second Dkk protein molecule); (iv) interaction with other extracellular proteins (*e.g.*, regulation of *wnt*-dependent cellular adhesion to extracellular matrix components); (v) binding to and eliminating an undesirable molecule (*e.g.*, a detoxifying activity or defense function); and/or (vi) an enzymatic activity, and can thus be used in, for example, (1)
25 modulation of cellular signal transduction, either *in vitro* or *in vivo* (*e.g.*, antagonism of the activity of members of the *wnt* family of secreted proteins or suppression of *wnt*-dependent signal transduction); (2) regulation of communication between cells (*e.g.*, regulation of *wnt*-dependent cell-cell interactions); (3) regulation of expression of genes whose expression is modulated by binding of Dkk (*e.g.*, hDkk-3) to a receptor; (4)
30 regulation of gene transcription in a cell involved in development or differentiation, either *in vitro* or *in vivo* (*e.g.*, induction of cellular differentiation); (5) regulation of gene transcription in a cell involved in development or differentiation, wherein at least one gene encodes a differentiation-specific protein; (6) regulation of gene transcription in a cell involved in development or differentiation, wherein at least one gene encodes a
35 second secreted protein; (7) regulation of gene transcription in a cell involved in development or differentiation, wherein at least one gene encodes a signal transduction molecule; (8) regulation of cellular proliferation, either *in vitro* or *in vivo* (*e.g.*, induction

of cellular proliferation or inhibition of proliferation, for example, inhibition of tumorigenesis (*e.g.*, inhibition of glioblastoma proliferation)); (9) formation and maintenance of ordered spatial arrangements of differentiated tissues in vertebrates, both adult and embryonic (*e.g.*, induction of head formation during vertebrate development or maintenance of hematopoietic progenitor cells); (10) modulation of cell death, such as stimulation of cell survival; (11) regulating cell migration; and/or (12) immune modulation.

Accordingly one embodiment of the present invention involves a method of use (*e.g.*, a diagnostic assay, prognostic assay, or a prophylactic/therapeutic method of treatment) wherein a molecule of the present invention (*e.g.*, a Dkk protein, Dkk nucleic acid, or a Dkk modulator) is used, for example, to diagnose, prognose and/or treat a disease and/or condition in which any of the aforementioned activities (*i.e.*, activities (i) - (vi) and (1) - (12) in the above paragraph) is indicated. In another embodiment, the present invention involves a method of use (*e.g.*, a diagnostic assay, prognostic assay, or a prophylactic/therapeutic method of treatment) wherein a molecule of the present invention (*e.g.*, a Dkk protein, Dkk nucleic acid, or a Dkk modulator) is used, for example, for the diagnosis, prognosis, and/or treatment of subjects, preferably a human subject, in which any of the aforementioned activities is pathologically perturbed. In a preferred embodiment, the methods of use (*e.g.*, diagnostic assays, prognostic assays, or prophylactic/therapeutic methods of treatment) involve administering to a subject, preferably a human subject, a molecule of the present invention (*e.g.*, a Dkk protein, Dkk nucleic acid, or a Dkk modulator) for the diagnosis, prognosis, and/or therapeutic treatment. In another embodiment, the methods of use (*e.g.*, diagnostic assays, prognostic assays, or prophylactic/therapeutic methods of treatment) involve administering to a human subject a molecule of the present invention (*e.g.*, a Dkk protein, Dkk nucleic acid, or a Dkk modulator).

Other embodiments of the invention pertain to the use of isolated nucleic acid molecules of the invention can be used, for example, to express Dkk or Dkk-related protein (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect Dkk or Dkk-related mRNA (*e.g.*, in a biological sample) or a genetic alteration in a Dkk or Dkk-related gene, and to modulate Dkk or Dkk-related activity, as described further below. In addition, the Dkk or Dkk-related proteins can be used to screen drugs or compounds which modulate the Dkk activity as well as to treat disorders characterized by insufficient or excessive production of Dkk or Dkk-related protein or production of Dkk or Dkk-related protein forms which have decreased or aberrant activity compared to Dkk or Dkk-related wild type protein (*e.g.*, developmental disorders or proliferative diseases such as cancer as well as diseases, ocular disorders

(*e.g.*, blindness) conditions or disorders characterized by abnormal cell differentiation and/or survival, an abnormal extracellular structure, or an abnormality in a defense mechanism). Moreover, the antibodies of the invention can be used to detect and isolate Dkk or Dkk-related proteins, regulate the bioavailability of Dkk or Dkk-related proteins, and modulate Dkk or Dkk-related activity. The term "an aberrant activity", as applied to an activity of a protein such as Dkk (*e.g.*, hDkk-3), refers to an activity which differs from the activity of the wild-type or native protein or which differs from the activity of the protein in a healthy subject. An activity of a protein can be aberrant because it is stronger than the activity of its native counterpart. Alternatively, an activity can be aberrant because it is weaker or absent related to the activity of its native counterpart. An aberrant activity can also be a change in an activity. For example an aberrant protein can interact with a different protein relative to its native counterpart. A cell can have an aberrant Dkk (*e.g.*, hDkk-3) activity due to overexpression or underexpression of the gene encoding Dkk.

A. Screening Assays:

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules or other drugs) which bind to Dkk or Dkk-related proteins or have a stimulatory or inhibitory effect on, for example, Dkk or Dkk-related expression or activity. Modulators can include, for example, agonists and/or antagonists. The term "agonist", as used herein, is meant to refer to an agent that mimics or upregulates (*e.g.* potentiates or supplements) a Dkk or Dkk-related (*e.g.*, hDkk-3) bioactivity. An agonist can be a compound which mimics a bioactivity of a Dkk or Dkk-related protein, such as transduction of a signal from a Dkk receptor, by, *e.g.*, interacting with a hDkk-3 receptor. An agonist can also be a compound that upregulates expression of a Dkk or Dkk-related gene. An agonist can also be a compound which modulates the expression or activity of a protein which is located downstream, for example, of a Dkk receptor, thereby mimicking or enhancing the effect of binding of Dkk to a Dkk receptor.

"Antagonist" as used herein is meant to refer to an agent that inhibits, decreases or suppresses a bioactivity (*e.g.*, hDkk-3). An antagonist can be a compound which decreases signalling from a Dkk or Dkk-related protein, *e.g.*, a compound that is capable of binding to hDkk-3 or to a hDkk-3 receptor. A preferred antagonist inhibits the interaction between a Dkk or Dkk-related protein and another molecule, such as a Dkk receptor. Alternatively, an antagonist can be a compound that downregulates expression

of a Dkk or Dkk-related gene. An antagonist can also be a compound which modulates the expression or activity of a protein which is located downstream of a Dkk receptor, thereby antagonizing the effect of binding of Dkk to a Dkk receptor.

In one embodiment, the invention provides assays for screening candidate or test
5 compounds which bind to or modulate the activity of a Dkk or Dkk-related protein or polypeptide or biologically active portion thereof. In another embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a Dkk receptor. The test compounds of the present invention can be
10 obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide
15 oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med.*
20 *Chem.* 37:2678; Cho *et al.*, (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor
25 (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In one embodiment, an assay is a cell-based assay in which a cell which
30 expresses a Dkk receptor on the cell surface is contacted with a test compound and the ability of the test compound to bind to a Dkk receptor determined. The cell, for example, can be of mammalian origin or a yeast cell. Determining the ability of the test compound to bind to a Dkk receptor can be accomplished, for example, by coupling the
35 test compound with a radioisotope or enzymatic label such that binding of the test compound to the Dkk receptor can be determined by detecting the labeled compound in

a complex. For example, test compounds can be labeled with ^{125}I , ^{35}S , ^{14}C , or ^3H , either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, test compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a test compound to interact with a Dkk receptor without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a test compound with a Dkk receptor without the labeling of either the test compound or the receptor. McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912. As used herein, a "microphysiometer" (*e.g.*, Cytosensor™) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between ligand and receptor.

In a preferred embodiment, the assay comprises contacting a cell which expresses a Dkk receptor on the cell surface with a Dkk protein or biologically-active portion thereof, to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a Dkk receptor, wherein determining the ability of the test compound to interact with a Dkk receptor comprises determining the ability of the test compound to preferentially bind to the Dkk receptor as compared to the ability of Dkk, or a biologically active portion thereof, to bind to the receptor.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a Dkk target molecule with a test compound and determining the ability of the test compound to modulate (*e.g.* stimulate or inhibit) the activity of the Dkk target molecule. Determining the ability of the test compound to modulate the activity of a Dkk target molecule can be accomplished, for example, by determining the ability of the Dkk protein to bind to or interact with the Dkk target molecule.

Determining the ability of the Dkk protein to bind to or interact with a Dkk target molecule can be accomplished by one of the methods described above for determining direct binding. In a preferred embodiment, determining the ability of the Dkk protein to bind to or interact with a Dkk target molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target molecule can be determined by detecting induction of a cellular second messenger of the target (*i.e.* intracellular Ca^{2+} , diacylglycerol, IP_3 , etc.), detecting catalytic/enzymatic activity of the target an appropriate substrate, detecting the induction of a reporter gene (comprising

a Dkk-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a cellular response, for example, development, differentiation or rate of proliferation.

In yet another embodiment, an assay of the present invention is a cell-free assay
5 in which a Dkk or Dkk-related protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the Dkk or Dkk-related protein or biologically active portion thereof is determined. Binding of the test compound to the Dkk or Dkk-related protein can be determined either directly or indirectly as described above. In a preferred embodiment, the assay includes contacting
10 the Dkk or Dkk-related protein or biologically active portion thereof with a known compound which binds Dkk or the Dkk-related protein to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a Dkk or Dkk-related protein, wherein determining the ability of the test compound to interact with a Dkk or Dkk-related protein comprises
15 determining the ability of the test compound to preferentially bind to Dkk or a Dkk-related protein or biologically active portion thereof as compared to the known compound.

In another embodiment, the assay is a cell-free assay in which a Dkk or Dkk-related protein or biologically active portion thereof is contacted with a test compound
20 and the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the Dkk or Dkk-related protein or biologically active portion thereof is determined. Determining the ability of the test compound to modulate the activity of a Dkk or Dkk-related protein can be accomplished, for example, by determining the ability of the Dkk or Dkk-related protein to bind to a target molecule (*e.g.*, a Dkk-target molecule) by one
25 of the methods described above for determining direct binding. Determining the ability of the Dkk or Dkk-related protein to bind to a target molecule can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA). Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705. As used herein, "BIA" is a technology for
30 studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore™). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In an alternative embodiment, determining the ability of the test compound to modulate the activity of a Dkk or Dkk-related protein can be accomplished by
35 determining the ability of the Dkk or Dkk-related protein to further modulate the activity of a target molecule (*e.g.*, a Dkk-target molecule). For example, the catalytic/enzymatic

activity of the target molecule on an appropriate substrate can be determined as previously described.

In yet another embodiment, the cell-free assay involves contacting a Dkk or Dkk-related protein or biologically active portion thereof with a known compound which binds the Dkk or Dkk-related protein to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the Dkk or Dkk-related protein, wherein determining the ability of the test compound to interact with the Dkk or Dkk-related protein comprises determining the ability of the Dkk or Dkk-related protein to preferentially bind to or modulate the activity of a target molecule (*e.g.*, a Dkk target molecule).

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with upstream or downstream elements. Accordingly, in an exemplary screening assay of the present invention, the compound of interest is contacted with a Dkk (*e.g.*, hDkk-3) protein or a Dkk (*e.g.*, hDkk-3) binding partner, *e.g.*, a receptor. The receptor can be soluble or the receptor can be present on a cell surface. To the mixture of the compound and the Dkk protein or Dkk binding partner is then added a composition containing a Dkk binding partner or a Dkk protein, respectively. Detection and quantification of complexes of Dkk proteins and Dkk binding partners provide a means for determining a compound's efficacy at inhibiting (or potentiating) complex formation between Dkk and a binding partner. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified Dkk polypeptide or binding partner is added to a composition containing the Dkk binding partner or Dkk polypeptide, and the formation of a complex is quantitated in the absence of the test compound.

The cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of isolated proteins (*e.g.* Dkk proteins or biologically active portions thereof or Dkk target molecules). In the case of cell-free assays in which

a membrane-bound form an isolated protein is used (*e.g.*, a Dkk target molecule or receptor) it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the isolated protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton[®] X-100, Triton[®] X-114, Thesit[®], Isotridecypoly(ethylene glycol ether)_n, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

10 In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either Dkk, a Dkk-related protein or a target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to a Dkk or Dkk-related protein, or interaction of a Dkk or Dkk-related
15 protein with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-transferase/
20 Dkk fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or Dkk protein, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological
25 conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of Dkk binding or activity determined using standard techniques.

30 Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either a Dkk protein, Dkk-related protein, or a Dkk target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated protein or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (*e.g.*, biotinylation kit,
35 Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with Dkk, Dkk-related protein, or target molecules but which do not interfere with binding of the protein to its

transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo*, forming a Dkk-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the Dkk or Dkk-related protein.

This invention further pertains to novel agents identified by the above-described screening assays and to processes for producing such agents by use of these assays. Accordingly, in one embodiment, the present invention includes a compound or agent obtainable by a method comprising the steps of any one of the aforementioned screening assays (*e.g.*, cell-based assays or cell-free assays). For example, in one embodiment, the invention includes a compound or agent obtainable by a method comprising contacting a cell which expresses a target molecule with a test compound and the determining the ability of the test compound to bind to, or modulate the activity of, the target molecule. In another embodiment, the invention includes a compound or agent obtainable by a method comprising contacting a cell which expresses a target molecule with a Dkk or Dkk-related protein or biologically-active portion thereof, to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with, or modulate the activity of, the target molecule. In another embodiment, the invention includes a compound or agent obtainable by a method comprising contacting a Dkk or Dkk-related protein or biologically active portion thereof with a test compound and determining the ability of the test compound to bind to, or modulate (*e.g.*, stimulate or inhibit) the activity of, the Dkk or Dkk-related protein or biologically active portion thereof. In yet another embodiment, the present invention includes a compound or agent obtainable by a method comprising contacting a Dkk or Dkk-related protein or biologically active portion thereof with a known compound which binds the Dkk or Dkk-related protein to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with, or modulate the activity of the Dkk or Dkk-related protein.

Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent

identified as described herein (*e.g.*, a Dkk modulating agent, an antisense Dkk nucleic acid molecule, a Dkk-specific antibody, or a Dkk-binding partner) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal
5 model to determine the mechanism of action of such an agent.

The present invention also pertains to uses of novel agents identified by the above-described screening assays for diagnoses, prognoses, and treatments as described herein. Accordingly, it is within the scope of the present invention to use such agents in the design, formulation, synthesis, manufacture, and/or production of a drug or
10 pharmaceutical composition for use in diagnosis, prognosis, or treatment, as described herein. For example, in one embodiment, the present invention includes a method of synthesizing or producing a drug or pharmaceutical composition by reference to the structure and/or properties of a compound obtainable by one of the above-described screening assays. For example, a drug or pharmaceutical composition can be
15 synthesized based on the structure and/or properties of a compound obtained by a method in which a cell which expresses a target molecule (*e.g.*, a Dkk target molecule) is contacted with a test compound and the ability of the test compound to bind to, or modulate the activity of, the target molecule is determined. In another exemplary embodiment, the present invention includes a method of synthesizing or producing a
20 drug or pharmaceutical composition based on the structure and/or properties of a compound obtainable by a method in which a Dkk or Dkk-related protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to, or modulate (*e.g.*, stimulate or inhibit) the activity of, the Dkk or Dkk-related protein or biologically active portion thereof is determined.

25

B. Detection Assays

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) map their
30 respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

35

1. Chromosome Mapping

Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. This process is

called chromosome mapping. Accordingly, portions or fragments of the Dkk or Dkk-related nucleotide sequences, described herein, can be used to map the location of the Dkk or Dkk-related genes on a chromosome. The mapping of the Dkk or Dkk-related sequences to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, Dkk or Dkk-related genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the Dkk or Dkk-related nucleotide sequences. Computer analysis of the Dkk or Dkk-related sequences can be used to predict primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the Dkk or Dkk-related sequences will yield an amplified fragment.

Somatic cell hybrids are prepared by fusing somatic cells from different mammals (*e.g.*, human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio P. *et al.*, (1983) *Science* 220:919-924). Somatic cell hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the Dkk or Dkk-related nucleotide sequences to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a 90, 1p, or 1v sequence to its chromosome include *in situ* hybridization (described in Fan, Y. *et al.*, (1990) *PNAS*, 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to chromosome specific cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been

blocked in metaphase by a chemical such as colcemid that disrupts the mitotic spindle. The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence
5 as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques
10 (Pergamon Press, New York 1988).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to
15 noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in V. McKusick, Mendelian Inheritance in
20 Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between a gene and a disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. *et al.* (1987) *Nature*, 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and
25 unaffected with a disease associated with a Dkk or Dkk-related gene, can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or
30 translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

35 2. Tissue Typing

The Dkk or Dkk-related sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for

example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the Dkk or Dkk-related nucleotide sequences described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this manner, can provide unique individual identifications, as each individual will have a unique set of such DNA sequences due to allelic differences. The sequences of the present invention can be used to obtain such identification sequences from individuals and from tissue. The Dkk or Dkk-related nucleotide sequences of the invention uniquely represent portions of the human genome. Allelic variation occurs to some degree in the coding regions of these sequences, and to a greater degree in the noncoding regions. It is estimated that allelic variation between individual humans occurs with a frequency of about once per each 500 bases. Each of the sequences described herein can, to some degree, be used as a standard against which DNA from an individual can be compared for identification purposes. Because greater numbers of polymorphisms occur in the noncoding regions, fewer sequences are necessary to differentiate individuals. The noncoding sequences of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, can comfortably provide positive individual identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding amplified sequence of 100 bases. If predicted coding sequences, such as those in SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:15 or SEQ ID NO:22 are used, a more appropriate number of primers for positive individual identification would be 500-2,000.

If a panel of reagents from Dkk or Dkk-related nucleotide sequences described herein is used to generate a unique identification database for an individual, those same reagents can later be used to identify tissue from that individual. Using the unique identification database, positive identification of the individual, living or dead, can be made from extremely small tissue samples.

3. Use of Partial Dkk or Dkk-related Sequences in Forensic Biology

DNA-based identification techniques can also be used in forensic biology.

Forensic biology is a scientific field employing genetic typing of biological evidence found at a crime scene as a means for positively identifying, for example, a perpetrator of a crime. To make such an identification, PCR technology can be used to amplify DNA sequences taken from very small biological samples such as tissues, *e.g.*, hair or skin, or body fluids, *e.g.*, blood, saliva, or semen found at a crime scene. The amplified sequence can then be compared to a standard, thereby allowing identification of the origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide reagents, *e.g.*, PCR primers, targeted to specific loci in the human genome, which can enhance the reliability of DNA-based forensic identifications by, for example, providing another "identification marker" (*i.e.* another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions of SEQ ID NOs:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the Dkk nucleotide sequences or portions thereof, *e.g.*, fragments derived from the noncoding regions of SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, or SEQ ID NO:20, having a length of at least 20 bases, preferably at least 30 bases.

The Dkk or Dkk-related nucleotide sequences described herein can further be used to provide polynucleotide reagents, *e.g.*, labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, *e.g.*, brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such Dkk or Dkk-related probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, *e.g.*, Dkk or Dkk-related primers or probes can be used to screen tissue culture for contamination (*i.e.* screen for the presence of a mixture of different types of cells in a culture).

C. Predictive Medicine:

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically.

Accordingly, one aspect of the present invention relates to diagnostic assays for determining Dkk or Dkk-related protein and/or nucleic acid expression as well as Dkk or Dkk-related activity, in the context of a biological sample (*e.g.*, blood, serum, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant Dkk or Dkk-related expression or activity, such as aberrant cell proliferation, differentiation, and/or survival resulting for example in a neurodegenerative disease (*e.g.*, Alzheimer's disease, Parkinson's disease, Huntington's chorea, amyotrophic lateral sclerosis and the like, as well as spinocerebellar degenerations) or cancer (for example, cancers of the epithelia (*e.g.*, carcinomas of the pancreas, stomach, liver, secretory glands (*e.g.*, adenocarcinoma) bladder, lung, breast, skin (*e.g.*, malignant melanoma), reproductive tract including prostate gland, ovary, cervix and uterus); cancers of the hematopoietic and immune system (*e.g.*, leukemias and lymphomas); cancers of the central nervous, brain system and eye (*e.g.*, gliomas, glioblastoma, neuroblastoma and retinoblastoma); and cancers of connective tissues, bone, muscles and vasculature (*e.g.*, sarcomas)). The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with Dkk or Dkk-related protein, nucleic acid expression or activity. For example, mutations in a Dkk or Dkk-related gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with Dkk or Dkk-related protein, nucleic acid expression or activity.

Another aspect of the invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of Dkk or Dkk-related in clinical trials.

These and other agents are described in further detail in the following sections.

1. Diagnostic Assays

An exemplary method for detecting the presence or absence of Dkk or Dkk-related protein or nucleic acid in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting Dkk or Dkk-related protein or nucleic acid (*e.g.*, mRNA, genomic DNA) that encodes Dkk or Dkk-related protein such that the presence of Dkk or Dkk-related protein or nucleic acid is detected in the biological sample. A preferred agent for detecting Dkk or Dkk-related mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to Dkk or Dkk-related mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length Dkk nucleic acid, such as the

nucleic acid of SEQ ID NO: 1, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:13, SEQ ID NO:20, the DNA insert of the plasmid deposited with ATCC as Accession Number 98452, the DNA insert of the plasmid deposited with ATCC as Accession Number 98633, or the nucleotide sequence of the DNA insert of the plasmid deposited with
5 ATCC as Accession Number _____, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to Dkk or Dkk-related mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

10 A preferred agent for detecting Dkk or Dkk-related protein is an antibody capable of binding to the protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')₂) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody
15 by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.
20 The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is; the detection method of the invention can be used to detect Dkk or Dkk-related mRNA, protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of Dkk or Dkk-related mRNA include
25 Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of Dkk or Dkk-related protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of Dkk or Dkk-related genomic DNA include Southern hybridizations. Furthermore, *in vivo* techniques for detection of Dkk or Dkk-related protein include
30 introducing into a subject a labeled antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In one embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the
35 test subject or genomic DNA molecules from the test subject. A preferred biological sample is a serum sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting Dkk or Dkk-related protein, mRNA, or genomic DNA, such that the presence of Dkk or Dkk-related protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of Dkk or Dkk-related protein, mRNA or genomic DNA in the control sample with the presence of Dkk or Dkk-related protein, mRNA or genomic DNA in the test sample.

The invention also encompasses kits for detecting the presence of Dkk or a Dkk-related protein in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting Dkk or Dkk-related protein or mRNA in a biological sample; means for determining the amount of Dkk or Dkk-related protein or mRNA in the sample; and means for comparing the amount of Dkk or Dkk-related protein or mRNA in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect Dkk or Dkk-related protein or nucleic acid.

2. Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant Dkk expression or activity. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with Dkk or Dkk-related protein, nucleic acid expression or activity such as a proliferative disorder, a differentiative or developmental disorder, a hematopoietic disorder as well as diseases, conditions or disorders characterized by abnormal cell survival, abnormal extracellular structure, or an abnormality in a defense mechanism. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing a differentiative or proliferative disease (*e.g.*, cancer). Thus, the present invention provides a method for identifying a disease or disorder associated with aberrant Dkk or Dkk-related expression or activity in which a test sample is obtained from a subject and Dkk or Dkk-related protein or nucleic acid (*e.g.*, mRNA, genomic DNA) is detected, wherein the presence of Dkk or Dkk-related protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant Dkk or Dkk-related expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue.

Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant Dkk or Dkk-related expression or activity. For example, such methods can be used to determine whether a subject can be effectively treated with an agent for a disorder, such as a proliferative disorder, a differentiative or developmental disorder, a hematopoietic disorder, as well disorders characterized by abnormal cell survival, an abnormal extracellular structure, or an abnormality in a defense mechanism. Alternatively, such methods can be used to determine whether a subject can be effectively treated with an agent for a differentiative or proliferative disease (*e.g.*, cancer). Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant Dkk or Dkk-related expression or activity in which a test sample is obtained and Dkk or Dkk-related protein or nucleic acid expression or activity is detected (*e.g.*, wherein the abundance of Dkk or Dkk-related protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant Dkk or Dkk-related expression or activity.)

The methods of the invention can also be used to detect genetic alterations in a Dkk or Dkk-related gene, thereby determining if a subject with the altered gene is at risk for a disorder characterized by aberrant development, aberrant cellular differentiation, aberrant cellular proliferation or an aberrant hematopoietic response. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding a Dkk or Dkk-related-protein, or the mis-expression of the Dkk or Dkk-related gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a Dkk or Dkk-related gene; 2) an addition of one or more nucleotides to a Dkk or Dkk-related gene; 3) a substitution of one or more nucleotides of a Dkk or Dkk-related gene, 4) a chromosomal rearrangement of a Dkk or Dkk-related gene; 5) an alteration in the level of a messenger RNA transcript of a Dkk or Dkk-related gene, 6) aberrant modification of a Dkk or Dkk-related gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a Dkk or Dkk-related gene, 8) a non-wild type level of a Dkk or Dkk-related-protein, 9) allelic loss of a Dkk or Dkk-related gene, and 10) inappropriate post-translational modification of a Dkk or Dkk-related-protein. As described herein, there are a large number of assay techniques known in the art which can be used for detecting

alterations in a Dkk or Dkk-related gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 5 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *PNAS* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the Dkk or Dkk-related-gene (see Abравaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682). This method can include the steps of collecting 10 a sample of cells from a patient, isolating nucleic acid (*e.g.*, genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a Dkk or Dkk-related gene under conditions such that hybridization and amplification of the Dkk or Dkk-related-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size 15 of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication 20 (Guatelli, J.C. *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D.Y. *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. *et al.*, 1988, *Bio/Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection 25 schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a Dkk or Dkk-related gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one 30 or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage 35 site.

In other embodiments, genetic mutations in a Dkk or Dkk-related gene can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high

density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M.T. *et al.* (1996) *Human Mutation* 7: 244-255; Kozal, M.J. *et al.* (1996) *Nature Medicine* 2: 753-759). For example, genetic mutations in Dkk can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M.T. *et al. supra.* Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the Dkk or Dkk-related gene and detect mutations by comparing the sequence of the sample Dkk or Dkk-related sequence with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxim and Gilbert ((1977) *PNAS* 74:560) or Sanger ((1977) *PNAS* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No. WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the Dkk or Dkk-related gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes of formed by hybridizing (labeled) RNA or DNA containing the wild-type Dkk or Dkk-related sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the

site of mutation. See, for example, Cotton *et al.* (1988) *Proc. Natl Acad Sci USA* 85:4397; Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

5 In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in Dkk cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 10 15:1657-1662). According to an exemplary embodiment, a probe based on a Dkk sequence, *e.g.*, a wild-type Dkk sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

15 In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in Dkk or Dkk-related genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA*: 86:2766, see also Cotton (1993) *Mutat Res* 285:125-144; and Hayashi (1992) *Genet Anal Tech Appl* 9:73-79). Single-stranded DNA fragments of sample and control Dkk or Dkk-related nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet* 7:5).

25 30 In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys Chem* 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl Acad. Sci USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, *e.g.*, in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a Dkk gene.

Furthermore, any cell type or tissue in which Dkk or a Dkk-related sequence is expressed may be utilized in the prognostic assays described herein.

3. Monitoring of Effects During Clinical Trials

Monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of Dkk or Dkk-related molecule (*e.g.*, modulation of cellular signal transduction, regulation of gene transcription in a cell involved in development or differentiation, regulation of cellular proliferation) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a

screening assay as described herein to increase Dkk or Dkk-related gene expression, protein levels, or upregulate Dkk or Dkk-related activity, can be monitored in clinical trials of subjects exhibiting decreased Dkk or Dkk-related gene expression, protein levels, or downregulated Dkk or Dkk-related activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease Dkk or Dkk-related gene expression, protein levels, or downregulate Dkk or Dkk-related activity, can be monitored in clinical trials of subjects exhibiting increased Dkk or Dkk-related gene expression, protein levels, or upregulated Dkk or Dkk-related activity. In such clinical trials, the expression or activity of Dkk or Dkk-related and, preferably, other genes that have been implicated in, for example, a proliferative disorder can be used as a "read out" or markers of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including Dkk and Dkk-related genes, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) which modulates Dkk or Dkk-related activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on proliferative disorders, developmental or differentiative disorder, hematopoietic disorder as well disorders characterized by abnormal cell differentiation and/or survival, an abnormal extracellular structure, or an abnormality in a defense mechanism, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of Dkk, Dkk-related, and other genes implicated in the proliferative disorder, developmental or differentiative disorder, hematopoietic disorder as well as disorders characterized by abnormal cell differentiation and/or survival, an abnormal extracellular structure, or an abnormality in a defense mechanism, respectively. The levels of gene expression (*i.e.*, a gene expression pattern) can be quantified by Northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of Dkk, Dkk-related, or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a Dkk or Dkk-related protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-

administration samples from the subject; (iv) detecting the level of expression or activity of the Dkk or Dkk-related protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the Dkk or Dkk-related protein, mRNA, or genomic DNA in the pre-administration sample with the Dkk or Dkk-related protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of Dkk or Dkk-related nucleic acid or protein to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of Dkk or Dkk-related nucleic acid or protein to lower levels than detected, *i.e.* to decrease the effectiveness of the agent. According to such an embodiment, Dkk or Dkk-related expression or activity may be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

15 C. Methods of Treatment:

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant Dkk or Dkk-related expression or activity. With regards to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers the study of how a patient's genes determine his or her response to a drug (*e.g.*, a patient's "drug response phenotype", or "drug response genotype".) Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the Dkk or Dkk-related molecules of the present invention or Dkk or Dkk-related modulators according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

35 1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant Dkk or Dkk-related expression or

activity, by administering to the subject an agent which modulates Dkk or Dkk-related expression or at least one Dkk or Dkk-related activity. Subjects at risk for a disease which is caused or contributed to by aberrant Dkk or Dkk-related expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the Dkk or Dkk-related aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of Dkk or Dkk-related aberrancy, for example, an agonist or antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein. The prophylactic methods of the present invention are further discussed in the following subsections.

2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating Dkk or Dkk-related expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of Dkk or Dkk-related protein activity associated with the cell. An agent that modulates Dkk or Dkk-related protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring target molecule of a Dkk or Dkk-related protein, a peptide, a Dkk or Dkk-related peptidomimetic, or other small molecule. In one embodiment, the agent stimulates one or more Dkk or Dkk-related protein activity. Examples of such stimulatory agents include active Dkk or Dkk-related protein and a nucleic acid molecule encoding Dkk or Dkk-related that has been introduced into the cell. In another embodiment, the agent inhibits one or more Dkk or Dkk-related protein activity. Examples of such inhibitory agents include antisense Dkk or Dkk-related nucleic acid molecules and antibodies. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity of a Dkk or Dkk-related protein or nucleic acid molecule. The present invention also provides methods of modulating the function, morphology, proliferation, and/or differentiation of cells in the tissues in which a Dkk or Dkk-related protein or nucleic acid molecule is expressed. Alternatively, Dkk or Dkk-related polypeptides, nucleic acids, and modulators thereof, can be used to treat disorders associated with abnormal or aberrant metabolism or function of cells in the tissues in which the Dkk or Dkk-related protein or nucleic acid molecule is expressed.

For example, tissues in which Dkk-3 is expressed include embryonic eye, bone,

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and cartilage, fetal brain, lung, and kidney, and adult heart (in particular, atrioventricular valves and atrial myocytes), eye (in particular, the integrating bipolar and ganglion cells of the retina, the ciliary body, and lens epithelium), brain (in particular, neurons of the cortex and hippocampus), placenta, lung, and skeletal muscle. Accordingly, Dkk-3

5 polypeptides, nucleic acids, or modulators thereof, can be used to treat cardiovascular disorders, such as ischemic heart disease (*e.g.*, angina pectoris, myocardial infarction, and chronic ischemic heart disease), hypertensive heart disease, pulmonary heart disease, valvular heart disease (*e.g.*, rheumatic fever and rheumatic heart disease, endocarditis, mitral valve prolapse, and aortic valve stenosis), congenital heart disease

10 (*e.g.*, valvular and vascular obstructive lesions, atrial or ventricular septal defect, and patent ductus arteriosus), or myocardial disease (*e.g.*, myocarditis, congestive cardiomyopathy, and hypertrophic cardiomyopathy).

In another embodiment, Dkk-3 polypeptides, nucleic acids, or modulators thereof, can be used to treat optic disorders such as diseases associated with amaurosis

15 (*e.g.*, a. fugax and a. albuminuric) diseases associated with amblyopia, glaucoma, optic neuropathy (*e.g.*, ischemic neuropathy, optic neuritis, and infiltrative neuropathy), ophthalmia (*e.g.*, o. catarrhal, trachoma, o. neuroparalytic, and conjunctiva), visual disorders resulting from systemic disease or disorders of other tissues (*e.g.*, diabetes mellitus, hyperthyroidism, and vitamin A or riboflavin deficiency), or tumors,

20 neoplasms, and metastases.

In another embodiment, Dkk-3 polypeptides, nucleic acids, or modulators thereof, can be used to treat disorders of the brain, such as cerebral edema, senile dementia of the Alzheimer type, epilepsy, amnesia, hydrocephalus, brain herniations, iatrogenic disease (due to, *e.g.*, infection, toxins, or drugs), inflammations (*e.g.*, bacterial

25 and viral meningitis, encephalitis, and cerebral toxoplasmosis), cerebrovascular diseases (*e.g.*, hypoxia, ischemia, and infarction, intracranial hemorrhage and vascular malformations, and hypertensive encephalopathy), and tumors (*e.g.*, neuroglial tumors, neuronal tumors, tumors of pineal cells, meningeal tumors, primary and secondary lymphomas, intracranial tumors, and medulloblastoma), and to treat injury or trauma to

30 the brain.

In another embodiment, Dkk-3 polypeptides, nucleic acids, or modulators thereof, can be used to treat placental disorders, such as toxemia of pregnancy (*e.g.*, preeclampsia and eclampsia), placentitis, or spontaneous abortion.

In another embodiment, Dkk-3 polypeptides, nucleic acids, or modulators

35 thereof, can be used to treat pulmonary disorders, such as atelectasis, pulmonary congestion or edema, chronic obstructive airway disease (*e.g.*, emphysema, chronic bronchitis, bronchial asthma, and bronchiectasis), diffuse interstitial diseases (*e.g.*,

sarcoidosis, pneumoconiosis, hypersensitivity pneumonitis, Goodpasture's syndrome, idiopathic pulmonary hemosiderosis, pulmonary alveolar proteinosis, desquamative interstitial pneumonitis, chronic interstitial pneumonia, fibrosing alveolitis, hamman-rich syndrome, pulmonary eosinophilia, diffuse interstitial fibrosis, Wegener's
5 granulomatosis, lymphomatoid granulomatosis, and lipid pneumonia), or tumors (*e.g.*, bronchogenic carcinoma, bronchioloalveolar carcinoma, bronchial carcinoid, hamartoma, and mesenchymal tumors).

In another embodiment, Dkk-3 polypeptides, nucleic acids, or modulators thereof, can be used to treat disorders of skeletal muscle, such as muscular atrophy (due
10 to, *e.g.*, denervation, malnutrition, loss of blood supply, or neuromuscular disease, *e.g.*, amyotonia congenita, amyotrophic lateral sclerosis of Charcot, and progressive muscular atrophy of Aran-Duchenne), myositis (due to, *e.g.*, bacterial, viral, fungal or parasitic infection), muscular dystrophies (*e.g.*, Duchenne type, Becker type, facioscapulohumeral, limb-girdle, myotonic dystrophy, and ocular myopathy),
15 myasthenia gravis, or tumors and tumor-like lesions of muscles (*e.g.*, traumatic myositis ossificans, desmoids, musculoaponeurotic fibromatosis, Dupuytren's contracture, nodular (pseudosarcomatous) fasciitis, rhabdomyoma, rhabdomyosarcoma, and granular cell myoblastomas).

Tissues in which Dkk-4 is expressed include cerebellum, activated human T-
20 lymphocytes, lung, and esophagus. Accordingly, in one embodiment, Dkk-4 polypeptides, nucleic acids, or modulators thereof, can be used to treat disorders of the cerebellum, such as disturbances of synergy (*e.g.*, asynergia or limb ataxia, dysmetria, decomposition of movement, hypermetria, hypometria, dysdiadochokinesia, hypotonia, tremor, dysarthria, nystagmus), disturbances of equilibrium (due to, *e.g.*, a lesion
25 involving the vestibulocerebellum), disturbances of gait stance, or tone (due to, *e.g.*, a lesion or degeneration of the spinocerebellum), or tumors (*e.g.*, astrocytoma and medulloblastoma).

In another embodiment, Dkk-4 polypeptides, nucleic acids, or modulators thereof, can be used to treat lymphocytic disorders, such as lymphopenia,
30 lymphocytosis, acute and chronic lymphadenitis, malignant lymphomas (*e.g.*, Non-Hodgkin's lymphomas, Hodgkin's lymphomas, leukemias, multiple myeloma, histiocytoses, and angioimmunoblastic lymphadenopathy).

In another embodiment, Dkk-4 polypeptides, nucleic acids, or modulators thereof, can be used to treat pulmonary disorders, such as atelectasis, pulmonary
35 congestion or edema, chronic obstructive airway disease (*e.g.*, emphysema, chronic bronchitis, bronchial asthma, and bronchiectasis), diffuse interstitial diseases (*e.g.*, sarcoidosis, pneumoconiosis, hypersensitivity pneumonitis, Goodpasture's syndrome,

idiopathic pulmonary hemosiderosis, pulmonary alveolar proteinosis, desquamative interstitial pneumonitis, chronic interstitial pneumonia, fibrosing alveolitis, hamman-rich syndrome, pulmonary eosinophilia, diffuse interstitial fibrosis, Wegener's granulomatosis, lymphomatoid granulomatosis, and lipid pneumonia), or tumors (e.g.,
5 bronchogenic carcinoma, bronchioloalveolar carcinoma, bronchial carcinoid, hamartoma, and mesenchymal tumors).

In another embodiment, Dkk-4 polypeptides, nucleic acids, or modulators thereof, can be used to treat esophageal disorders, such as neuromuscular disturbances (e.g., achalasia, annular narrowings, Schatzki's rings, hiatal hernia, Mallory-Weiss
10 syndrome), esophagitis (due to e.g., bacteremia, viremia, fungal infections, uremia, graft-versus-host disease, chemotherapy, radiation, and prolonged gastric intubation), diverticula (e.g., Zenker's diverticulum), systemic sclerosis, varices (due to, e.g., portal hypertension, systemic amyloidosis and sarcoidosis), or tumors or neoplasms (e.g., leiomyoma, fibromas, lipomas, hemangiomas, lymphangiomas, squamous papillomas,
15 adenocarcinomas and undifferentiated carcinomas, and sarcomas).

Dkk-1 is highly expressed, for example, in placenta. Accordingly, Dkk-1 polypeptides, nucleic acids, or modulators thereof, can be used to treat placental disorders, such as toxemia of pregnancy (e.g., preeclampsia and eclampsia), placentitis, or spontaneous abortion.

20 Tissues in which Dkk-2 is expressed include, for example, heart, brain, placenta, lung, and skeletal muscle. Accordingly, Dkk-2 polypeptides, nucleic acids, or modulators thereof, can be used to treat cardiovascular disorders, such as ischemic heart disease (e.g., angina pectoris, myocardial infarction, and chronic ischemic heart disease), hypertensive heart disease, pulmonary heart disease, valvular heart disease (e.g.,
25 rheumatic fever and rheumatic heart disease, endocarditis, mitral valve prolapse, and aortic valve stenosis), congenital heart disease (e.g., valvular and vascular obstructive lesions, atrial or ventricular septal defect, and patent ductus arteriosus), or myocardial disease (e.g., myocarditis, congestive cardiomyopathy, and hypertrophic
30 cardiomyopathy).

In another embodiment, Dkk-2 polypeptides, nucleic acids, or modulators thereof, can be used to treat disorders of the brain, such as cerebral edema, senile dementia of the Alzheimer type, epilepsy, amnesia, hydrocephalus, brain herniations, iatrogenic disease (due to, e.g., infection, toxins, or drugs), inflammations (e.g., bacterial and viral meningitis, encephalitis, and cerebral toxoplasmosis), cerebrovascular diseases
35 (e.g., hypoxia, ischemia, and infarction, intracranial hemorrhage and vascular malformations, and hypertensive encephalopathy), and tumors (e.g., neuroglial tumors, neuronal tumors, tumors of pineal cells, meningeal tumors, primary and secondary

lymphomas, intracranial tumors, and medulloblastoma), and to treat injury or trauma to the brain.

In another embodiment, Dkk-2 polypeptides, nucleic acids, or modulators thereof, can be used to treat placental disorders, such as toxemia of pregnancy (*e.g.*,
5 preeclampsia and eclampsia), placentitis, or spontaneous abortion.

In another embodiment, Dkk-2 polypeptides, nucleic acids, or modulators thereof, can be used to treat pulmonary disorders, such as atelectasis, pulmonary congestion or edema, chronic obstructive airway disease (*e.g.*, emphysema, chronic
10 bronchitis, bronchial asthma, and bronchiectasis), diffuse interstitial diseases (*e.g.*, sarcoidosis, pneumoconiosis, hypersensitivity pneumonitis, Goodpasture's syndrome, idiopathic pulmonary hemosiderosis, pulmonary alveolar proteinosis, desquamative
15 interstitial pneumonitis, chronic interstitial pneumonia, fibrosing alveolitis, hamman-rich syndrome, pulmonary eosinophilia, diffuse interstitial fibrosis, Wegener's granulomatosis, lymphomatoid granulomatosis, and lipid pneumonia), or tumors (*e.g.*,
15 bronchogenic carcinoma, bronchioloalveolar carcinoma, bronchial carcinoid, hamartoma, and mesenchymal tumors).

In another embodiment, Dkk-2 polypeptides, nucleic acids, or modulators thereof, can be used to treat disorders of skeletal muscle, such as muscular atrophy (due to, *e.g.*, denervation, malnutrition, loss of blood supply, or neuromuscular disease, *e.g.*,
20 amyotonia congenita, amyotrophic lateral sclerosis of Charcot, and progressive muscular atrophy of Aran-Duchenne), myositis (due to, *e.g.*, bacterial, viral, fungal or parasitic infection), muscular dystrophies (*e.g.*, Duchenne type, Becker type, facioscapulohumeral, limb-girdle, myotonic dystrophy, and ocular myopathy),
25 myasthenia gravis, or tumors and tumor-like lesions of muscles (*e.g.*, traumatic myositis ossificans, desmoids, musculoaponeurotic fibromatosis, Dupuytren's contracture, nodular (pseudosarcomatous) fasciitis, rhabdomyoma, rhabdomyosarcoma, and granular cell myoblastomas).

Soggy-1 is expressed in, for example, testis (*e.g.*, spermatogenic epithelium of the seminiferous tubules, spermatogonia) and in embryonic developing dorsal root
30 ganglia, cartilage primordium of the nasal septum, and the eye. Accordingly, Soggy-1 polypeptides, nucleic acids, or modulators thereof, can be used to treat testicular disorders, such as unilateral testicular enlargement (*e.g.*, nontuberculous, granulomatous orchitis), inflammatory diseases resulting in testicular dysfunction (*e.g.*, gonorrhea and mumps), and tumors (*e.g.*, germ cell tumors, interstitial cell tumors, androblastoma,
35 testicular lymphoma and adenomatoid tumors). In another embodiment, Soggy-1 polypeptides, nucleic acids, or modulators thereof, can be used to treat infertility due to, for example, spermatogenetic failure.

In one aspect, the above-described methods involve administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) Dkk or Dkk-related expression or activity. In another embodiment, the method involves administering a Dkk or Dkk-
5 related protein or nucleic acid molecule as therapy to compensate for reduced or aberrant Dkk or Dkk-related expression or activity.

A preferred embodiment of the present invention involves a method for treatment of a disease or disorder associated with a Dkk or Dkk-related protein which includes the step of administering a therapeutically effective amount of an antibody to a Dkk or Dkk-
10 related protein to a subject. As defined herein, a therapeutically effective amount of antibody (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg,
15 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a
20 subject is treated with antibody in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody used for treatment may increase or decrease over the course of a particular treatment.
25 Changes in dosage may result from the results of diagnostic assays as described herein.

Stimulation of Dkk or Dkk-related activity is desirable in situations in which Dkk or Dkk-related activity is abnormally downregulated and/or in which increased Dkk or Dkk-related activity is likely to have a beneficial effect. Likewise, inhibition of Dkk or Dkk-related activity is desirable in situations in which Dkk or Dkk-related activity is
30 abnormally upregulated and/or in which decreased Dkk or Dkk-related activity is likely to have a beneficial effect. One example of such a situation is where a subject has a disorder characterized by aberrant development or cellular differentiation. Another example of such a situation is where the subject has a proliferative disease (*e.g.*, cancer) or a neurogenerative disorder. Yet another example of such a situation is where it is
35 desirable to achieve tissue regeneration in a subject (*e.g.*, where a subject has undergone brain or spinal cord injury and it is desirable to regenerate neuronal tissue in a regulated manner.)

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Accordingly, in one embodiment, the disease is a disease characterized by an abnormal cell proliferation, differentiation, and/or survival. For example, the disease can be a hyper-or hypoproliferative disease. The invention also provides methods for treating diseases characterized by an abnormal cell proliferation, differentiation, and/or survival in a subject, which are not characterized by an abnormal Dkk or Dkk-related activity (*e.g.*, hDkk-3 activity). In fact, since Dkk is likely to be capable of modulating the proliferative state of a cell (*i.e.*, state of proliferation, differentiation, and or survival of a cell), Dkk can regulate disease wherein the abnormal proliferative state of a cell results from a defect other than an abnormal Dkk activity.

Hyperproliferative diseases can be treated with Dkk or Dkk-related (*e.g.*, hDkk-3) therapeutics include neoplastic and hyperplastic diseases, such as various forms of cancers and leukemias, and fibroproliferative disorders. Other hyperproliferative diseases that can be treated or prevented with the subject Dkk or Dkk-related therapeutics (*e.g.* hDkk-3 therapeutics) include malignant conditions, premalignant conditions, and benign conditions. The condition to be treated or prevented can be a solid tumor, such as a tumor arising in an epithelial tissue. Accordingly, treatment of such a cancer could comprise administration to the subject of a Dkk or Dkk-related therapeutic decreasing the interaction of Dkk with a Dkk receptor. Other cancers that can be treated or prevented with a Dkk or Dkk-related protein include cancers of the epithelia (*e.g.*, carcinomas of the pancreas, kidney, stomach, colon, esophagus liver, secretory glands (*e.g.*, adenocarcinoma) bladder, lung, breast, skin (*e.g.*, malignant melanoma, seminoma squamous adenocarcinoma), reproductive tract including prostate gland, testis, ovary, cervix and uterus); cancers of the hematopoietic and immune system (*e.g.*, leukemias and lymphomas); cancers of the central nervous, brain system and eye (*e.g.*, malignant astrocytoma, gliomas, neuroblastoma and retinoblastoma); and cancers of connective tissues, bone, heart, muscles and vasculature (*e.g.*, sarcomas, for example, osteosarcoma). Additional solid tumors within the scope of the invention include those that can be found in a medical textbook.

The condition to be treated or prevented can also be a soluble tumor, such as leukemia, either chronic or acute, including chronic or acute myelogenous leukemia, chronic or acute lymphocytic leukemia, promyelocytic leukemia, monocytic leukemia, myelomonocytic leukemia, and erythroleukemia. Yet other proliferative disorders that can be treated with a Dkk or Dkk-related therapeutic of the invention include heavy chain disease, multiple myeloma, lymphoma, *e.g.*, Hodgkin's lymphoma and non-Hodgkin's lymphoma, and Waldenstroem's macroglobulemia.

Diseases or conditions characterized by a solid or soluble tumor can be treated by administering a Dkk or Dkk-related therapeutic either locally or systemically, such that

aberrant cell proliferation is inhibited or decreased. Methods for administering the compounds of the invention are further described below.

The invention also provides methods for preventing the formation and/or development of tumors. For example, the development of a tumor can be preceded by
5 the presence of a specific lesion, such as a pre-neoplastic lesion, *e.g.*, hyperplasia, metaplasia, and dysplasia, which can be detected, *e.g.*, by cytologic methods. Such lesions can be found, *e.g.*, in epithelial tissue. Thus, the invention provides a method for inhibiting progression of such a lesion into a neoplastic lesion, comprising administering
10 therapeutic sufficient to inhibit progression of the preneoplastic lesion into a neoplastic lesion.

The invention also provides for methods for treating or preventing diseases or conditions in which proliferation of cells is desired. For example, Dkk or Dkk-related
15 therapeutics can be used to stimulate tissue repair or wound healing, such as after surgery or to stimulate tissue healing from burns. Other diseases in which proliferation of cells is desired are hypoproliferative diseases, *i.e.*, diseases characterized by an abnormally low proliferation of certain cells.

In yet another embodiment, the invention provides a method for treating or preventing diseases or conditions characterized by aberrant cell differentiation.
20 Accordingly, the invention provides methods for stimulating cellular differentiation in conditions characterized by an inhibition of normal cell differentiation which may or may not be accompanied by excessive proliferation. Alternatively, Dkk or Dkk-related therapeutics can be used to inhibit differentiation of specific cells.

In a preferred method, the aberrantly proliferating and/or differentiating cell is a
25 cell present in the nervous system. A role for Dkk in the nervous system is suggested at least in part from the fact that human Dkk-3 is expressed in human fetal brain. Accordingly, the invention provides methods for treating diseases or conditions associated with a central or peripheral nervous system. For example, the invention provides methods for treating lesions of the nervous system associated with an aberrant
30 proliferation, differentiation or survival of any of the following cells: cells of the central nervous system including neurons and glial cells (*e.g.*, astrocytes and oligodendrocytes) and supporting cells of peripheral neurons (*e.g.*, Schwann cells and satellite cells). Disorders of the nervous system include, but are not limited to: spinal cord injuries, brain injuries, brain tumors (*e.g.*, astrocytic tumors, for example, astrocytomas and
35 glioblastomas), lesions associated with surgery, ischemic lesions, malignant lesions, infectious lesions, degenerative lesions (*e.g.*, Parkinson's disease, Alzheimer's disease, Huntington's chorea, amyotrophic lateral sclerosis), demyelinating diseases (*e.g.*,

multiple sclerosis, human immunodeficiency associated myelopathy, transverse myelopathy, progressive multifocal leukoencephalopathy, pontine myelinolysis), motor neuron injuries, progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile muscular atrophy, progressive bulbar paralysis of childhood (*i.e.*, Fazio-Londe syndrome), poliomyelitis, and hereditary motorsensory neuropathy (*i.e.*, Charcot-Marie-Tooth disease).

In another embodiment, the invention provides a method for enhancing the survival and/or stimulating proliferation and/or differentiation of cells and tissues *in vitro*. In a preferred embodiment, Dkk or Dkk-related therapeutics are used to promote tissue regeneration and/or repair (*e.g.*, to treat nerve injury). For example, tissues from a subject can be obtained and grown *in vitro* in the presence of a Dkk or Dkk-related therapeutic, such that the tissue cells are stimulated to proliferate and/or differentiate. The tissue can then be readministered to the subject.

Among the approaches which may be used to ameliorate disease symptoms involving an aberrant Dkk or Dkk-related activity and/or an abnormal cell proliferation, differentiation, and/or survival, are, for example, antisense, ribozyme, and triple helix molecules described above. Examples of suitable compounds include the antagonists, agonists or homologues described in detail above.

Yet other Dkk or Dkk-related therapeutics consist of a first peptide comprising a Dkk or Dkk-related peptide capable of binding to a Dkk receptor, and a second peptide which is cytotoxic. Such therapeutics can be used to specifically target and lyse cells expressing or overexpressing a receptor for Dkk.

3. Pharmacogenomics

The Dkk or Dkk-related molecules of the present invention, as well as agents, or modulators which have a stimulatory or inhibitory effect on Dkk or Dkk-related activity (*e.g.*, Dkk or Dkk-related gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) disorders (*e.g.*, proliferative or developmental disorders) associated with aberrant Dkk or Dkk-related activity. In conjunction with such treatment, pharmacogenomics (*i.e.*, the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer a Dkk or Dkk-related molecule or Dkk or Dkk-related modulator as well as tailoring the dosage and/or

therapeutic regimen of treatment with a Dkk or Dkk-related molecule or Dkk or Dkk-related modulator.

Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See *e.g.*, Eichelbaum, M., *Clin Exp Pharmacol Physiol*, 1996, 23(10-11) :983-985 and Linder, M.W., *Clin Chem*, 1997, 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare genetic defects or as naturally-occurring polymorphisms. For example, glucose-6-phosphate dehydrogenase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association", relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (*e.g.*, a "bi-allelic" gene marker map which consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants.) Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high resolution map can be generated from a combination of some ten-million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a "SNP" is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process, however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

Alternatively, a method termed the "candidate gene approach", can be utilized to identify genes that predict drug response. According to this method, if a gene that encodes a drugs target is known (*e.g.*, a Dkk protein or Dkk receptor of the present invention), all common variants of that gene can be fairly easily identified in the

population and it can be determined if having one version of the gene versus another is associated with a particular drug response.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (*e.g.*, N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug. These polymorphisms are expressed in two phenotypes in the population, the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Alternatively, a method termed the "gene expression profiling", can be utilized to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (*e.g.*, a Dkk molecule or Dkk modulator of the present invention) can give an indication whether gene pathways related to toxicity have been turned on.

Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a Dkk molecule or Dkk or Dkk-related modulator, such as a modulator identified by one of the exemplary screening assays described herein.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

EXAMPLES

The invention is based, at least in part, on the discovery of a family of genes encoding human cysteine-rich secreted proteins which are related to *Xenopus* Dickkopf (Dkk) proteins. This family includes hDkk-1, hDkk-2, hDkk-3, and hDkk-4. hDkks 1-4 contain two highly conserved cysteine-rich domains (CRDs), the most C-terminal of which demonstrates similarity to the colipase protein family. The invention is based also in part on the discovery of a family of Dkk-related proteins, referred to as Soggy proteins, as well as the genes encoding Soggy proteins. Soggy-1 is a novel secreted protein which is related to the N-terminal region of Dkk-3 but lacks CRDs. The following examples illustrate the structure and function of each of these novel human secreted proteins.

Example 1: Isolation And Characterization of Human hDkk-3 cDNA

In this example, the isolation and characterization of the gene encoding human Dkk-3 (also referred to as "hDkk-3", "Cysteine Rich Secreted Protein-1", "CRSP-1" "CRISPY-1" or "TANGO 59") is described.

Isolation of a Human Dkk-3 cDNA

The invention is based at least in part on the discovery of a human gene encoding a secreted protein, referred to herein as human Dickkopf-3 (hDkk-3). A partial cDNA was isolated using a Signal Sequence Trap method. This methodology takes advantage of the fact that molecules such as Dkk have an amino terminal signal sequence which directs certain secreted and membrane-bound proteins through the cellular secretory apparatus.

Briefly, a randomly primed cDNA library using mRNA prepared from human fetal brain tissue (Clontech, Palo Alto CA) was made by using the Stratagene-ZAP-cDNA Synthesis™ kit, (catalog #20041). The cDNA was ligated into the mammalian expression vector pTrap adjacent to a cDNA encoding placental alkaline phosphatase lacking a secretory signal. The plasmids were transformed into *E. coli* and DNA was prepared using the Wizard™ DNA purification kit (Promega). DNA was transfected into COS-7 cells with lipofectamine™ (Gibco-BRL). After 48 hours incubation the COS cell supernatants were assayed for alkaline phosphatase on a Wallac Micro-Beta scintillation counter using the Phospha-Light™ kit (Tropix Inc. Catalog #BP300). The individual plasmid DNAs scoring positive in the COS cell Alkaline Phosphatase secretion assay were further analyzed by DNA sequencing using standard procedures.

Using a partial cDNA isolated by the above-described method (clone Amhb3c2), a full length cDNA encoding human Dkk-3 was isolated from a lambda Ziplox™ human fetal brain cDNA library using conventional hybridization techniques (Sambrook *et al.*, *supra*). The nucleotide sequence encoding the full length human Dkk-3 protein is shown in Figure 1 and is set forth as SEQ ID NO: 1. The full length protein encoded by this nucleic acid is comprised of about 350 amino acids and has the amino acid sequence shown in Figure 1 and set forth as SEQ ID NO:2. The coding portion (open reading frame) of SEQ ID NO:1 is set forth as SEQ ID NO:3. DNA for the clone Fmhb059 was deposited with the ATCC as Accession No. 98452.

Analysis of Human hDkk-3

Determination of the hydrophobicity profile of human Dkk-3 having the amino acid sequence set forth in SEQ ID NO:2 indicated the presence of a hydrophobic region from about amino acid 1 to about amino acid 22 of SEQ ID NO:2. Further analysis of the amino acid sequence SEQ ID NO:2 using a signal peptide prediction program predicted the presence of a signal peptide from about amino acid 1 to about amino acid 22 of SEQ ID NO:2. Accordingly, the mature hDkk-3 protein includes about 328 amino acids spanning from about amino acid 23 to about amino acid 350 of SEQ ID NO:2. The presence of the signal sequence, in addition to the fact that hDkk-3 has been identified using a Signal Sequence Trap system, indicates that hDkk-3 is a secreted protein. Furthermore, the prediction of such a signal peptide and signal peptide cleavage site can be made, for example, utilizing the computer algorithm SIGNALP (Nielsen, *et al.*, (1997) *Protein Engineering* 10:1-6).

Examination of the cDNA sequence depicted in Figure 1 shows that human Dkk-3 is particularly rich in cysteine residues. As shown in Figure 1, hDkk-3 contains 20 cysteine residues located between amino acid 147 and amino acid 284 of SEQ ID NO: 2. This region has been termed the cysteine-rich region. These cysteine residues can form 10 disulfide bridges.

A BLAST search (Altschul *et al.*, (1990) *J. Mol. Biol.* 215:403) of the nucleotide and the amino acid sequences of hDkk-3 has revealed that hDkk-3 is similar to a chicken cDNA encoding a protein of unknown function having GenBank Accession No. D26311. This cDNA was isolated from a chicken lens cDNA library and was shown to be expressed in lens fibers and lens epithelium, but not in neural retina nor in liver cells. (Sawada *et al.*, (1996) *Int. J. Dev. Biol.* 40:531). hDkk-3 and the chicken protein have 56% amino acid sequence identity and 72% amino acid sequence similarity. The amino acid sequence similarity between the chicken protein and human Dkk-3 is particularly high in the cysteine-rich domain of hDkk-3 which is located between amino acids 147

and 284 of SEQ ID NO: 2. In particular, the 20 cysteine residues of hDkk-3 located in this region are present in the chicken protein.

Two genes recently identified in a screen for suppressors of glioblastoma formation (Ligon *et al.* (1997) *Oncogene* 14:1075-1081) also show homology to hDkk-3. These genes, RIG ("Regulated In Glioblastoma") and RIG-like 7-1 (GenBank Accession Nos. U32331 and AF034208, respectively) were identified in a differential screen for mRNAs regulated by the introduction of a normal copy of chromosome 10 into a glioblastoma cell line harboring a deletion in chromosome 10 that promotes tumorigenesis. A schematic diagram summarizing the relationship between the sequences of the hDkk-3 and the RIG genes is presented as Figure 12. The indicated region of identity between hDkk-3 and RIG comprises a short portion of the 3' UTR of the human Dkk-3 mRNA (*e.g.*, RIG mRNA is ~100% identical to residues 2479 to 2153 of SEQ ID NO:1). RIG-like 7-1 is homologous to hDkk-3 across a longer region (*e.g.*, 97% identical from about nucleotides 316 to 2438 of SEQ ID NO:1) although the encoded RIG-like 7-1 protein lacks the Dkk N-terminal signal sequence and is not therefore predicted to be a secreted protein. These data associate hDkk-3 with human glioblastoma and suggest that hDkk-3 may be important in the suppression of the tumorigenic phenotype. A role in glioblastoma is also consistent with the high level of hDkk-3 mRNA expression observed in human brain tissue. In addition, the co-localization of the hDkk-3, RIG and RIG-like genes to a region of chromosome 11 (11p15.1) implicated in the development of human malignant astrocytoma (Ligon *et al.*, *supra*) further indicates a role for these genes in tumorigenesis.

Human hDkk-3 protein has also some amino acid sequence similarity to metallothionein, particularly in the cyteine-rich domain.

Tissue Distribution of hDkk-3 mRNA

For Northern blots, all hybridizations were to Clontech Multiple Tissue Northern Blots and were performed in ExpressHyb solution (Clontech) for 1-20 hours. All probes were prepared by random primed radiolabelling (Prime-It, Stratagene). Blots were washed sequentially to a final stringency of 0.2x SSC/0.2% SDS and exposed to autoradiographic film. Hybridizations of a control β -actin cDNA probe consistently demonstrated even loading of the Northern blots. The results of hybridization of the probe to various mRNA samples are described below.

Hybridization of a Clontech Fetal Multiple Tissue Northern (MTN) blot (Clontech, LaJolla, CA) containing RNA from fetal brain, lung, liver, and kidney indicated the presence of high levels of hDkk-3 mRNA (~2.5kb) in fetal brain, lung, and

slightly lower levels of hDkk-3 mRNA in fetal kidney. However, no significant level of hDkk-3 mRNA was found in fetal liver.

Hybridization of a Clontech human Multiple Tissue Northern (MTN) blot (Clontech, LaJolla, CA) containing RNA from adult heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas with a human Dkk-3 probe indicated the presence of high levels of hDkk-3 mRNA in heart, slightly lower levels in brain, and much lower levels in placenta and lung. Some hDkk-3 mRNA was also found in adult skeletal muscle. However, no significant levels of hDkk-3 mRNA was observed in adult liver, kidney, or pancreas. Interestingly, the chicken gene which is homologous to hDkk-3 was not expressed at detectable levels in liver either (Sawada *et al.*, (1996) *Int. J. Dev. Biol.* 40:531).

Further hybridization of a Clontech human Multiple Tissue Northern (MTN) blot (Clontech, LaJolla, CA) including RNA from bone marrow, adrenal gland, trachea, lymph node, spinal cord, thyroid, and stomach revealed high levels of expression of hDkk-3 in mRNA isolated from adult spinal cord, and lower level expression in adrenal gland, trachea, thyroid, and stomach.

Thus, hDkk-3 is expressed in a tissue specific manner, with the strongest expression observed in brain, heart; and spinal cord.

Example 2: Isolation And Characterization of mDkk-3 cDNA

In this example, the isolation and characterization of the gene encoding murine Dkk-3 (also referred to as "mDkk-3", "murine Cysteine Rich Secreted Protein-1", "murine CRSP-1" or "murine CRISPY-1") is described.

Identification of a Murine Dkk-3 cDNA

A full length mDkk-3 cDNA was identified by comparison of the hDkk-3 sequence to a proprietary EST Database using the BLAST-X algorithm. A single clone identified in a adult mouse brain cDNA library was obtained and sequenced fully. DNA for the clone Fmmb059s was deposited with the ATCC as Accession No. 98634. mDkk-3 is predicted to have a signal peptide from residues 1 to 23 of SEQ ID NO:17, cleavage of which results in a mature protein having 326 amino acids in length corresponding to amino acids 24 to 349 of SEQ ID NO:17.

Tissue Distribution of mDkk-3 mRNA

To determine the expression pattern of mDkk-3, *in situ* hybridization was performed as follows. Normal mouse embryos and adult mouse tissues were collected from C57BL/6 mice, embedded in TissueTek™ O.C.T Compound (Sakura Finetek

U.S.A., Inc., Torrance, CA), frozen on dry ice, and stored at -80°C. Cryostat serial sections (8µm) were thaw mounted on Superfrost Plus™ slides (VWR Scientific, West Chester, PA.) and air dried on a slide warmer at 40°C for 20 minutes. Sections were then fixed with 4% formaldehyde in DEPC treated 0.1 M phosphate-buffered saline (PBS, pH 7.5) at room temperature for 10 minutes and rinsed twice in DEPC-PBS. Sections were rinsed in 0.1 M triethanolamine-HCl (TEA, pH 8.0), incubated in 0.25% acetic anhydride-TEA for 10 minutes and rinsed in DEPC-2X SSC (standard sodium citrate). Sections were dehydrated through a series of graded ethanols, incubated in 100% chloroform for 5 minutes, rinsed in 100% and 95% ethanol for 1 minutes and air dried.

Antisense and sense RNA transcripts were prepared by *in vitro* transcription (Riboprobe Gemini System™, Promega) of PCR amplified cDNA templates. Template amplification primers were as follows;

mDkk-3 forward 5'-CAGTGAGTGCTGTGGAGACC-3' (SEQ ID NO:30), and

reverse 5'-TCTTCAGTCAGGCTCCTCTC-3' (SEQ ID NO:31).

Probes were labeled with ³⁵S-UTP (NEN) and purified on G-25 spin columns (Pharmacia). The hybridization cocktail contained: 50% formamide, 10% dextran sulfate, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium thiosulfate, 1X Denhardt's solution, 0.6 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 100 mM dithiothreitol (DTT), 0.1 mg/ml sheared salmon sperm, 50 µg/ml yeast tRNA, 0.5 mg/ml yeast total RNA, and ³⁵S-UTP labeled probe at a concentration of 5 X 10⁷ c.p.m./100µl of final hybridization solution; 100 µl of hybridization solution was put on each section. The sections were then covered with a glass coverslip and incubated in a humidified chamber at 55°C for 18 h. After hybridization, slides were washed with 2 X SSC. Sections were then sequentially incubated at 37°C in TNE (a solution containing 10 mM Tris-HCl (pH 7.6), 500 mM NaCl, and 1 mM EDTA), for 10 minutes, in TNE with 10ug/ml RNase A for 30 minutes, and finally in TNE for 10 minutes. Slides were then rinsed with 2 X SSC at room temperature, washed in 2 X SSC at 50°C for 1 h, 0.2 X SSC at 55°C for 1h, and 0.2 X SSC at 60°C for 1 h. Sections were dehydrated with a series of graded concentrations of ethanol 0.3 M ammonium acetate, air dried and exposed to Kodak Biomax MR™ scientific imaging film for 6 days at room temperature.

mDkk-3 expression in the brain was found to be highly localized to the cortex and hippocampus but was not observed in the dentate gyrus. Higher power magnification confirmed the *mDkk-3* mRNA was localized to neurons within these structures. In the adult eye, *mDkk-3* mRNA was found to be highly expressed in the retina, ciliary body, and lens epithelium. Expression in the retina was localized to the integrating bipolar and ganglion cells. In adult heart, *mDkk-3* was detected in the

atrioventricular valves and also in myocytes of the atria. Expression was highly restricted to the atria and noticeably absent from ventricular tissue. High level expression of mDkk3 mRNA was also observed in developing eye, bone and cartilage in day 14 embryos. These findings corroborate and extend the northern analysis of hDkk-3 mRNA expression in human tissues and also suggest that Dkk-3 may play a role in bone and ocular physiology in addition to functions in neural and cardiac tissues.

Example 3: Secretion and Post-Translational Modification of Dkk-3

This example describes the secretion and post-translational modification (*e.g.*, glycosylation and processing) of hDkk-3 as well as methods for small and large scale purification of hDkk-3.

hDkk-3 Expression Constructs

Expression constructs for two forms of hDkk-3 were prepared using the mammalian expression vector pMET-stop. Form-1 comprised a cDNA incorporating the complete 350aa hDkk-3 protein coding sequence (hDkk-3flag.long) and form-2 comprised the entire hDkk-3 protein coding sequence except for the final 18 amino acids (hDkk-3flag.short). A C-terminal sequence encoding the FLAG epitope (DYKDDDDK) (SEQ ID NO:19) was added to both hDkk-3 forms for ease of detection and purification. hDkk-3flag cDNAs were generated by PCR from a full length hDkk-3 cDNA template and ligated into pMET-stop using EcoR1 and Sal1 restriction sites.

Trial Transfection - Small Scale Expression

Expression constructs for hDkk-3flag.long and hDkk-3flag.short were transfected into 293T cells using 10 μ l of lipofectamine (GIBCO/BRL) and 2 μ g of DNA per well of a 6-well plate of cells which were 70-80% confluent. After 5 hours at 37°C, cells were fed with 1ml of 20%FCS/DMEM. After incubation overnight at 37°C, cells were conditioned in 1ml OptiMEM for 48 hours at 37°C. Samples of supernatant and cell pellets were solubilized in boiling SDS-PAGE gel buffer, run out on a 4-20% SDS-PAGE gel, transferred to a nylon membrane and probed with the anti-FLAG monoclonal antibody M2. Samples from both supernatant and pellet samples showed significant immunoreactivity within a molecular weight range of 40-65 kDa on autoradiographic film using a HRP conjugated secondary antibody and ECL detection reagents. Thus, both forms of hDkk-3 tested are secreted from 293T cells thereby confirming experimentally that hDkk-3 is a secreted protein. It should be noted that the molecular weights of both forms of hDkk-3 tested are greater than predicted from the amino acid sequence, suggesting that the hDkk-3 proteins secreted by 293T cells may be

glycosylated. This is consistent with the presence of four potential sites for N-linked glycosylation in the hDkk-3 protein (*e.g.*, at about amino acids 96-99, 106-109, 121-124, and 204-207 of SEQ ID NO:2).

5 Deglycosylation of hDkk-3

Given the heterogenous nature of secreted human Dkk-3, the effect of N-Glycanase treatment on the mobility of secreted flag-tagged hDkk-3 was studied. Briefly, 1mL samples of 293T cell supernatants collected 72 hours after transfection with the appropriate constructs were incubated with 50 μ L anti-flag M2 agarose beads (Sigma) for 16hrs at 4°C. Beads were washed with PBS (pH7.4) containing, sequentially, 0.1%, 0.05% and 0.01% Triton X-100. The beads were resuspended in 20 μ L of 20mM sodium phosphate, pH 7.5, 50mM EDTA, 0.02% sodium azide, (incubation buffer) together with 0.5% SDS, 5% 2-mercaptoethanol and boiled for 2 minutes. The supernatant was split into equal 10 μ L aliquots which were diluted with 10 μ L incubation buffer, 5 μ L 5% NP-40 and then with either 5 μ L N-Glycanase (Oxford Glycosystems) in enzyme buffer (20mM Tris-HCl, 1mM EDTA, 50mM NaCl, 0.02% sodium azide pH 7.5) or with enzyme buffer alone as control. After 18 hours at 37°C, samples were boiled in equal volumes of SDS-PAGE buffer and analyzed by SDS-PAGE and Western blotting. For western analysis, samples were electroblotted onto PVDF (Novex) after SDS-PAGE on 4-20% gradient gels, probed with M2 anti-flag antibody (1:500, Sigma) followed by HRP conjugated sheep anti-mouse IgG (1:5000, Amersham), developed with chemiluminescent reagents (Renaissance, Dupont) and exposed to autoradiography film (Biomax MR2 film, Kodak).

Utilizing the above-described methodology, it was determined that hDkk-3 protein displayed a significant increase in mobility following N-Glycanase treatment. The major 45-65 kD form of soluble hDkk-3 was observed as two species of 45-55 and 40 kD following deglycosylation. This finding is consistent with the presence of multiple potential sites of N-linked glycosylation in the hDkk-3 protein. The reason for the heterogeneity of deglycosylated hDkk-3 reflects either proteolytic processing or incomplete removal of carbohydrate from one or more attachment sites. A 30 kD hDkk3 species was also observed in these experiments, the mobility of which was unaltered by N-Glycanase treatment. This form was only observed after overnight incubation of the samples and may be a non-specific degradation product.

35 Large Scale hDkk-3 Protein Production

For scale-up of hDkk-3flag.long protein expression, 30 x 150mM plates of 293T cells at 70-80% confluence were transfected with 27 μ g DNA, 100 μ l lipofectamine in

18ml OptiMEM for 5 hours at 37°C. 18 ml of 10%FCS/DMEM was added to each plate and incubated overnight at 37°C. 24 hours after the start of transfection, transfection supernatant was aspirated and 35 mls OptiMEM was added to each plate and the plates incubated at 37°C for 72 hours. Conditioned medium was harvested, spun at 4000 rpm
5 for 30 min. at 4°C, and filtered through a 0.45 micron filter unit. 1100 ml was passed over a 1.6 x 10 cm anti-FLAG M2 affinity column pre-equilibrated in PBS pH7.4 buffer at a flow rate of 2.0 ml per minute. After washing with 200 ml of PBS pH 7.4 buffer, bound material was eluted by a step of 200 mM Glycine pH 3.0 buffer and 0.5 ml fractions collected. Upon elution, a significant protein peak was detected by absorbance
10 at 280nm. Samples corresponding to conditioned medium, flow through and eluted fractions were analyzed by Coomassie blue and silver stained SDS-PAGE and by western blot analysis as described above. Significant immunoreactivity within a molecular weight range of 40-65 kDa was detected in conditioned medium and eluted
15 3flag.long protein bound to the affinity column specifically and was eluted efficiently by the described conditions. Coomassie blue staining of SDS-PAGE gels suggested that the predominant immunoreactive protein constituted >90% of the protein present in the bound and eluted protein peak. Peak fractions of eluted protein were pooled and dialysed against Phosphate Buffered Saline resulting in a 4 ml volume of recombinant
20 hDkk-3flag.long protein at a concentration of approximately 1mg/ml.

Example 4: Isolation and Characterization of hDkk-4

In this example, the isolation and characterization of the gene encoding human Dkk-4 (also referred to as "hDkk-4", "Cysteine Rich Secreted Protein-2", "CRSP-2" or
25 "CRISPY-2") is described.

Isolation and Analysis of a Human Dkk-4 cDNA

To identify novel proteins related to hDkk-3, the human Dkk-3 amino acid sequence was used to search the dbEST database using TBLASTN (WashUversion, 2.0,
30 BLOSUM62 search matrix). A dbEST clone with accession number AA565546 was identified as having homology to a portion of the hDkk-3 cDNA. This clone was obtained from the IMAGE consortium and sequenced fully to define the entire hDkk-4 sequence depicted in Figure 2.

Determination of the hydrophobicity profile of human Dkk-4 having the amino
35 acid sequence set forth in SEQ ID NO:5 indicated the presence of a hydrophobic region from about amino acid 1 to about amino acid 19 of SEQ ID NO:5. Further analysis of the amino acid sequence SEQ ID NO:5 using a signal peptide prediction program

predicted the presence of a signal peptide from about amino acid 1 to about amino acid 19 of SEQ ID NO:5. Accordingly, the mature hDkk-4 protein includes about 205 amino acids spanning from about amino acid 20 to about amino acid 224 of SEQ ID NO:5.

5 Tissue Distribution of hDkk-4

hDkk-4 mRNA was undetectable by Northern analysis in all adult and fetal human tissues examined. Accordingly, a survey was performed of a cDNA library panel by PCR with hDkk-4 specific PCR primers. Using such primers, products were identified in libraries prepared from cerebellum, activated human T-lymphocytes, lung and esophagus.

Secretion and Post-Translational Modification of human Dkk-4

Flag epitope-tagged human Dkk-4 protein was transiently overexpressed in 293T cells and analyzed as described previously for hDkk-3. Soluble hDkk-4 was consistently detected as three major immunoreactive species of approximately 40 kD [form (i)], 30-32 kD [form (ii)] and 15-17 kD [form (iii)]. Neither form (i), (ii) or (iii) was significantly affected by N-glycanase treatment, consistent with the absence of N-glycosylation sites from the protein.

To determine the possible cause of heterogeneity in the size of secreted hDkk-4, Edman N-terminal sequencing of anti-flag affinity purified material corresponding to bands (i), (ii) and (iii) was performed. Briefly, flag-tagged Dkk-4 protein was isolated by passing the conditioned media over an M2-biotin (Sigma)/streptavidin Poros column (2.1 X 30 mm, PE Biosystems); the column was then washed with PBS, pH 7.4 and flag-tagged protein eluted with 200 mM glycine, pH 3.0. Eluted fractions with 280nm absorbance greater than background were analyzed by SDS-PAGE and western blot. Purified Dkk-4 protein bound to PVDF membrane after SDS-PAGE and electroblotting was sequenced for N-terminal amino acid analysis on a PE Applied Biosystems Model 494 Procise instrument using Edman-based chemistry protein sequencing. The amino acid residues were analyzed by HPLC (Spherogel micro PTH 3-micron column) and determined by separation and peak height as compared to standards.

The N-terminal sequence of band (i) was found to be XVLDFNIRS (SEQ ID NO:34) which corresponds exactly to the predicted signal peptide cleavage site (between Ala-18 and Leu-19). Because the same band is identified by anti-flag antibodies, which recognize the C-terminal epitope tag, band (i) was thus identified as the full length, mature hDkk-4 protein. The band (iii) N-terminal sequence was found to be SQGRKGQEGS (SEQ ID NO:38) which corresponds to CRD-2 cleaved at the dibasic site Lys132/Lys133 (e.g., Lys113/Lys114 of the mature protein following cleavage of

the a 19 amino acid signal sequence or Lys 114/Lys 115 following cleavage of a 18 amino acid signal sequence). These data obtained for bands (i) and (iii) indicate clearly that hDkk4 is proteolytically processed by 293T cells, resulting in the release of CRD-2 (a 91 amino acid biologically-active fragment) from the full length protein.

5 Moreover, the three major species migrated similarly on SDS-PAGE conducted under either reducing or non-reducing conditions. Thus, each of the major C-terminal (anti-flag immunoreactive) hDkk-4 species exist as independent proteolytic fragments that are not covalently linked via disulfide bonds to other subunits or complex components when secreted from 293T cells.

10

Example 5: Isolation and Characterization of hDkk-1

In this example, the isolation and characterization of the gene encoding human Dkk-1 (also referred to as "hDkk-1", "Cysteine Rich Secreted Protein-3", "CRSP-3" or
15 "CRISPY-3") is described.

Identification of a Human Dkk-1 cDNA

Searching a proprietary database of EST information using the sequence of hDkk-3, an hDkk-1 partial sequence was found corresponding to a clone from a human
20 fetal kidney cDNA library having the identification code jthKb075a10. This clone was sequenced further and to define the entire hDkk-1 sequence depicted in Figure 3. DNA for the clone jthKb075a10 was deposited with the ATCC as Accession No. 98633. hDkk-1 has a predicted signal peptide from about amino acid residue 1 to 20 of SEQ ID NO:8, cleavage of which results in a mature protein having 246 amino acid residues in
25 length and corresponding to amino acid residues 21 to 266 of SEQ ID NO:8.

Tissue Distribution of hDkk-1

Northern blot analysis of various tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was performed as previously described
30 using a probe specific for hDkk-1. A ~1.8 kb hDkk1 mRNA was detected at high levels in human placenta, but not in other tissues tested.

Secretion and Post-Translational Modification of hDkk-1

Flag epitope-tagged human Dkk-1 protein was transiently overexpressed in 293T
35 cells and analyzed as described previously. hDkk-1 was efficiently secreted from mammalian cells and was readily detected in conditioned medium of transfected cells. Mature secreted hDkk-1 migrated with a molecular weight of approximately 42-50 kD.

Treatment with N-Glycanase had no significant effect on the mobility of soluble hDkk-1. Although hDkk-1 contains one potential site of N-linked glycosylation at its extreme C-terminus (*e.g.*, at amino acids 256-259 of SEQ ID NO8), this site is not conserved in *Xenopus* Dkk-1 (Glinka *et al.*, *supra*) and appears not to be a major site of carbohydrate addition in 293T cells.

Example 6: Isolation and Characterization of hDkk-2

In this example, the isolation and characterization of the gene encoding human Dkk-2 (also referred to as "hDkk-2", "Cysteine Rich Secreted Protein-4", "CRSP-4" or "CRISPY-4") is described.

Isolation of a Human Dkk-2 cDNA

Using the hDkk-3 sequence to query the dbEST database, a clone having similarity to a portion of hDkk-3 was identified having Accession No. W55979. This clone was subsequently obtained from the IMAGE consortium and sequenced to define a partial hDkk-2 sequence set forth as SEQ ID NO:10. This cDNA comprises a coding region from nucleotides 1-537, as well as 3' untranslated sequences (nucleotides 538 to 702). The coding region alone is set forth as SEQ ID NO:12. The predicted amino acid sequence corresponds to amino acids 1 to 179 of SEQ ID NO:11. A cDNA encoding full length hDkk-2 was isolated from a human fetal lung lambda Ziplox libraries by conventional plaque hybridization (Sambrook *et al.*, 1989) and fully sequenced. The full-length nucleotide sequence is set forth as SEQ ID NO:20 and the predicted amino acid sequence is set forth as SEQ ID NO:21. The coding region alone is set forth as SEQ ID NO:22. The predicted amino acid sequence corresponds to amino acids 1 to 259 of SEQ ID NO:21. DNA for the clone fthu133 was deposited with the ATCC as Accession No. _____. hDkk-2 has a predicted signal peptide from about amino acid residue 1 to 33 of SEQ ID NO:21, cleavage of which results in a mature protein having 226 amino acid residues in length and corresponding to amino acid residues 34 to 259 of SEQ ID NO:21.

Tissue Distribution of hDkk-2

Northern blot analysis of various tissues (*e.g.*, heart, brain, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, and peripheral blood leukocytes) was performed as previously described using a probe specific for hDkk-2. Of the tissues tested, hDkk-2 mRNA expression was highest in heart, brain, placenta,

lung, and skeletal muscle. hDkk-2 transcripts of approximately 4.0 and 4.5 kb were observed.

Secretion and Post-translational Modification of hDkk-2

5 Flag epitope-tagged human Dkk-2 protein was transiently overexpressed in 293T cells and analyzed as described previously. Soluble hDkk-2 was detected as a major species of 15-17 kD, closely similar in size to form (iii) of hDkk-4. Additional minor forms of hDkk-2 were also observed in certain experiments in the range of 20-21kD. Deglycosylation of hDkk-2 was not studied since the protein sequence lacks potential N-
10 glycosylation sites. By comparison with the data presented in Example 4 regarding the dibasic proteolytic cleavage site in the hDkk-4 protein sequences, it is predicted that the major 15-17 kD form of hDkk-2 detected in these experiments corresponds to CRD-2, as was the case for hDkk-4.

15

Example 7: Isolation of Soggy proteins

In this example, the isolation and characterization of the gene encoding human and murine Soggy-1 (also referred to as "Cysteine Rich Secreted Protein-N" or "CRISP-N") is described.

20

Identification of a Human and Murine Soggy-1 cDNAs

Human Soggy-1 was identified as a novel protein with similarity to the N-terminal domain of hDkk3. A human partial sequence was identified in the dbEST database for a clone having the accession number AA397836. This clone was obtained
25 from the IMAGE collection and sequenced fully to define the entire human Soggy-1 sequence depicted in Figure 7. Two murine partial sequences were likewise identified in the dbEST database. The clones were obtained from the IMAGE consortium and sequenced. The entire murine Soggy-1 sequence is depicted in Figure 8. Human and murine Soggy cDNAs encode proteins of 242aa and 230aa, respectively, and are
30 predicted to be secreted owing to the presence of N-terminal signal peptides. hSoggy-1 has a predicted signal peptide from about amino acid residue 1 to 30 of SEQ ID NO:14, cleavage of which results in a mature protein having 194 amino acid residues in length and corresponding to amino acid residues 31 to 224 of SEQ ID NO:14. mSoggy-1 has a predicted signal peptide from about amino acid residue 1 to 20 of SEQ ID NO:27,
35 cleavage of which results in a mature protein having 210 amino acid residues in length and corresponding to amino acid residues 21 to 230 of SEQ ID NO:27. Human and murine Soggy proteins display 59% overall identity although significant amino acid

identities are seen beyond this domain that extend into the CRDs of Dkk-3 (Figure 10). However, cysteine residues are not conserved within these domains and the residues shared by Soggy and Dkk-3 are poorly conserved in other Dkks indicating that the sequence relationship between these proteins is unique. Homology is most obvious within a 51 amino acid region in which 33% identity is observed between hSoggy, mSoggy, hDkk-3 and mDkk-3. This 51 amino acid domain is referred to herein as an SGY domain. Human and mouse Soggy-1 proteins each possess 2 sites of potential N-linked glycosylation which are within the SGY domain and are also conserved with Dkk3. (e.g., NNTL, corresponding to amino acid residues 97-100 of SEQ ID NO:14 and NKTG corresponding to amino acid residues 112-115 of SEQ ID NO:14). In contrast to other Dkks, the C-terminal domain of Soggy-1 shows no similarity to other protein sequences in the public databases nor does it contain any cysteine residues. Soggy was so named in view of its lack of CRDs compared to hDkk-3, which had been previously designated Cysteine Rich Secreted Protein-1 ("CRISPY-1").

Tissue Distribution of Soggy-1

To investigate Soggy-1 mRNA expression, a mouse cDNA probe was used on murine Northern blots. A 1kb mSoggy-1 mRNA was expressed at very high levels in testis and, interestingly, demonstrated transient expression during mouse embryogenesis. Soggy-1 mRNA, which was undetectable at day 7 of gestation, was transiently expressed at day 11 and day 15, after which the expression level declined to undetectable levels. Thus, mSoggy-1 displays a developmentally regulated pattern of expression.

In situ analysis was performed as described in Example 1. For detection of murine Soggy-1, the following primers were used:

mSoggy forward 5'-ACCTGCAATGTGTCGACTGAG-3' (SEQ ID NO:32), and
reverse 5'-CACTTACAGCTGTTGGGATG-3' (SEQ ID NO:33).

Consistent with the Northern analysis, very high level expression of Soggy-1 mRNA was observed by *in situ* analysis in adult testis. Upon high magnification, Soggy-1 mRNA was found to be expressed at high levels in the spermatogenic epithelium of the seminiferous tubules and in the spermatogonia at various stages of development. A series of sagittal sections of mouse embryos from E13.5 - E 17.5 and post-natal day 1.5 pups were also analyzed. In E15.5 embryos, Soggy-1 mRNA transcripts were localized to the developing dorsal root ganglia (DRGs) and also found in the cartilage primordium of the nasal septum. Soggy-1 expression was also seen in the eye from E13.5 to E16.5, as observed for mDkk-3. Expression of Soggy-1 mRNA

at various stages of development is consistent with the northern analysis described above and suggests that Soggy-1 may play a role in multiple stages of development.

Secretion and Post-Translational Modification of Soggy Proteins

- 5 Flag epitope-tagged human Soggy-1 protein was transiently overexpressed in 293T cells and analysed as previously described. hSoggy was efficiently secreted from transfected 293T cells and migrated with a molecular weight of approximately 40-50 kD. Given the heterogenous nature of secreted human Soggy-1, the effect of N-Glycanase treatment on the mobility of secreted flag-tagged hSoggy-1 was studied.
- 10 hSoggy displayed a 5-10 kD decrease in apparent molecular weight after N-Glycanase treatment, consistent with the presence of 2 potential sites of N-glycosylation in the protein.

Example 8: Structure of the Dkk Family proteins and Dkk-Related Proteins

- 15 The amino acid and nucleotide homology between Dkk family members and Dkk-related proteins is set forth in the following tables. Where indicated, mDkk-1 and xDkk-1 correspond to a murine and *Xenopus* proteins set forth in Glinks *et al., supra*, and having Accession Nos: AF030433 and AF030434, respectively. Likewise cDkk-3 has Accession No. D26311

20

Table II sets forth overall sequence identities as determined using the ALIGN program, (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4:

	hDkk-3	hDkk-4	hDkk-1	hDkk-2	mDkk-1	xDkk-1	CLFEST
hDkk-3	100	16.0	18.6	15.1	18.5	16.5	53.0
hDkk-4		100	33.7	35.2	32.6	33.7	16.2
hDkk-1			100	33.1	80.2	53.5	17.4
hDkk-2				100	30.5	33.7	12.5

25

Table III sets forth nucleic acid identities as determined using the using the Wilbur Lipman DNA alignment program, Ktuple: 3; Gap Penalty: 3; Window: 20:

	hDkk-3	hDkk-4	hDkk-1	hDkk-2	mDkk-1	xDkk-1	CLFEST
hDkk-3	100	30.0	37.2	34.7	31.5	45.4	58.8
hDkk-4		100	43.0	35.9	38.8	38.4	36.7
hDkk-1			100	59.3	66.4	53.7	32.1
hDkk-2				100	38.8	38.4	36.7

- 5 **Table IV** sets forth local amino acid identities as determined using the Smith-Waterman algorithm as implemented in the program Bestfit of the GCG package, with gap penalties of 8 for opening and 1 for extending:

hDkk-1								
mDkk-1	82							
xDkk-1	64	63						
hDkk-2	50	48	47					
hDkk-3	39	37	37	37				
mDkk-3	36	33	38	40	83			
cDkk-3	34	31	35	36	61	60		
hDkk-4	45	43	47	46	40	39	34	
	hDkk-1	mDkk-1	xDkk-1	hDkk-2	hDkk-3	mDkk-3	cDkk-3	hDkk-4

- 10 A multiple alignment of the amino acid sequences of hDkk-1, hDkk-2, hDkk-3, hDkk-4, mDkk-1, mDkk-3, xDkk-1, and cDkk-3 is shown in Figure 6. Predicted signal peptides are underlined, N-glycosylation sites are indicated by a thick bar, CRD-1 by an open box and CRD-2 by a shaded box. The proteolytic cleavage site within hDkk4 is indicated by a double asterisk. The domain structure of the full length human Dkk
- 15 proteins of the present invention as well as human Soggy are schematically illustrated in Figure 9. Signal peptides (darkened boxes), Cysteine Rich Domain 1 ("CRD-1") (also referred to as the "amino-terminal cysteine-rich domain"), Cysteine Rich Domain 2 ("CRD-2") (also referred to as the "carboxy-terminal cysteine-rich domain"), the soggy domain (SGY) within hDkk-3 and hSoggy-1, and sites of N-glycosylation are indicated.
- 20 As demonstrated at least in Figures 6 and 9, human Dkks 1 through 4 each possess an N-terminal signal peptide and contain two conserved cysteine-rich domains (CRDs) separated by a linker or spacer region. Each CRD possesses 10 conserved

cysteine residues. The second CRD has elsewhere been described as a colipase-like domain because the positions of the ten conserved cysteines in this domain have been shown to be closely similar to those in proteins of the colipase family (Aravind and Koonin, *supra*). Conservation of CRD-1 and CRD-2 suggests important functions for these domains. In contrast to the CRDs, the linker or spacer region that joins CRD-1 and CRD-2 is highly variable between hDkks, being notably larger in hDkk-1, -2 and -4 (50-55aa) as compared to Dkk-3 (12aa). Four potential sites of N-linked glycosylation are present in hDkk3 and are conserved in chicken and mouse Dkk-3. These sites are not conserved in other Dkk family members. hDkk1 possesses one potential N-glycosylation site located close to the C-terminus of the protein which is conserved in murine Dkk-1 but not in *Xenopus* Dkk-1 (Fig. 6). In addition, each hDkk possesses several potential dibasic proteolytic cleavage sites, suggesting the proteins may be subject to post-translational processing. hDkk3 is the most divergent of the four human Dkks and possesses an extended N-terminal unique region which precedes CRD-1 and an extended C-terminal unique region which is highly acidic.

Example 9: Effects of hDkks and Soggy on Wnt-induced axis duplication in *Xenopus* embryos

This Example describes the functional activities of the hDkk and Soggy proteins of the present invention.

Xenopus embryo culture and RNA microinjections

Eggs were obtained from *Xenopus* females injected with 700 units of human chorionic gonadotropin, fertilized *in vitro* and cultured in 0.1 x MMR (Newport and Kirschner (1982) *Cell* 30:675-686). Embryonic stages were determined according to Nieuwkoop and Faber (1967) *Normal table of Xenopus laevis (Daudin)* Amsterdam: North Holland Publ. All cDNAs were subcloned into pCS2 vector (Rupp *et al.* (1984) *Genes & Development* 8:1311-1323), and capped mRNAs were synthesized *in vitro* as described (Krieg and Melton (1984) *Nucleic Acids Res.* 12:7057-7070), using the Message Machine kit (Ambion). The following plasmids were used as templates for mRNA synthesis: hDkk-1-pCS2, hDkk-2-pCS2, hDkk-3-pCS2, hDkk-4-pCS2, hSoggy-pCS2, Xwnt8 (Christian *et al.*, (1991) *Development* 111:1045-1055), Xwnt2B (Landesman and Sokol (1997) *Mech. Dev.* 61:1199-209), Xwnt3a (Wolda *et al.* (1993) *Dev. Biol.* 155:46-5), Xfz8-pXT7 (Itoh *et al.* (1998) *Mech. Devel.* 74:145-157), Xdsh-pXT7 (Sokol, *et al.* (1995) *Mech. Devel.* 74:145-157). Protein expression from all pCS2-Dkk constructs was confirmed by *in vitro* transcription and translation (TNT,

Promega). For secondary axis induction, a single ventral blastomere of 4- or 8-cell embryos was injected with 10nl of a solution containing 2-4pg of Xwnt8 mRNA, 2.5-5pg of Xwnt3a mRNA or 10pg of Xwnt2B mRNA as described (Itoh *et al.* (1995) *supra*). The effect of Dkk RNAs was tested by coinjecting Wnt mRNAs with 2.5ng of hDkk mRNAs. For studies of Frizzled and Dhshevelled, 5ng Fz8 and 1ng Xdsh mRNAs were injected as indicated. After injections, embryos were cultured in 3% Ficoll 400 (Pharmacia), 0.5xMMR. Secondary axes were scored at stage 35 as complete, when they contained anterior neuroectodermal derivatives including pronounced cement gland and eyes, and as partial, when the secondary neural tube with melanocytes, but without head structures, was apparent.

Inhibition of Secondary Axis Induction by hDkk-1 and hDkk-4 in *Xenopus* Embryos

hDkk-1 or hDkk-2 mRNAs were coinjected with Xwnt8 mRNA into single ventral blastomeres of 4- or 8-cell embryos. Injected embryos were cultured for 2 days and secondary axes were scored based on external morphology. Xwnt8 injected embryos displayed complete axis duplication, which was inhibited by co-injection with mRNAs encoding hDkk-1 and hDkk-4. To determine whether hDkks interacted with specific Wnt ligands, several different Wnts were assayed in combination with hDkk-1 or hDkk-4 for secondary axis formation. hDkk-1 and hDkk-4 inhibited axis duplication in response to Xwnt3a and Xwnt2b in addition to Xwnt8. hDkk-1 consistently demonstrated stronger inhibition of Wnt signaling than hDkk-4. Thus, hDkk-1 and hDkk-4 do not show any clear selectivity for the Wnt ligands used in this study. This compares to the FRPs, which also show little specificity with respect to their ability to inhibit Wnts (Leyns *et al.* (1997) *supra*; Wang *et al.* (1997) *supra*; Salic *et al.* (1997) *supra*; Mayr *et al.* (1997) *supra*; Finch *et al.* (1997) *supra*).

To investigate the mechanism by which hDkk-1 and hDkk-4 inhibit Wnt signaling, Dkk mRNAs were coinjected with Xdsh, a downstream component of the Wnt signaling pathway (Itoh *et al.* (1998) *supra*). hDkks-1 and -4 did not block secondary axis formation by Xdsh, indicating that Dkks function upstream of, or parallel with, Xdsh signaling. Similar findings have been reported previously for xDkk-1 (Glinka *et al.* (1998) *supra*). It was also determined whether hDkks could antagonize signaling by *Xenopus* Frizzled-8 (Xfz8), which can also induce a secondary axis through Wnt signaling (Itoh *et al.* (1998) *supra*). Neither hDkk-1 or hDkk-4 inhibited the axis-inducing activity of Xfz8 mRNA. This data, taken together with the fact that hDkk-1 and hDkk-4 are secreted, indicate that Dkks antagonize Wnt signaling at a point upstream of Wnt receptors.

Assay for Inhibition of Secondary Axis Induction by hDkk-2, hDkk-3 and hSoggy-1 in *Xenopus* Embryos

hDkk-2, hDkk-3 or Soggy mRNAs were coinjected with Xwnt8 mRNA into single ventral blastomeres of 4- or 8-cell embryos and secondary axes were scored after
 5 two days as described for hDkk-1 and hDkk-4. Injection of mRNAs encoding hDkk-2, hDkk-3 or hSoggy-1 had no effect on Xwnt8-induced axis duplication. The ability of hDkk-2, hDkk-3 and hSoggy-1 to interact with specific Wnt ligands was also determined as described previously. hDkk-2, hDkk-3 and hSoggy-1 were inactive against each of the three Wnts tested. The lack of activity of hDkk-2, hDkk-3 and hSoggy-1 suggests
 10 that these proteins antagonize other members of the Wnt superfamily not tested here, or that they perform functions distinct from Wnt inhibition.

Example 10: Preparation of Antibodies Specific for hDkk and hSoggy Proteins

This example describes the making of polyclonal antibodies specific for hDkk-
 15 1, hDkk-4, hDkk-1, hDkk-2, and hSoggy-1.

Peptides were synthesized using Fmoc solid phase methodology utilizing MAP resin technology which increases the antigenic response (Tarn (1988) *Proc. Natl. Acad. Sci. USA* 85:5409-5413. For each protein, the peptides used for immunization
 20 are listed below:

	<u>hDkk-3</u>	
	peptide #44	FREVEELMEDTQHKL
	peptide #46	GSFMEEVRQELEDLE
	<u>hDkk-4</u>	
25	peptide #91	HAEGTTGHPVQENQP
	<u>hDkk-1</u>	
	peptide #93	GNKYQTIDNYQPYP
	<u>hDkk-2</u>	
	peptide #56	GHYSNHDLGWQNLGR
30	<u>hSoggy-1</u>	
	peptide #58	LQAIRDGLRKGTHKD

Peptides were designed to meet at least the following criteria: (1) not included within the cysteine-rich domain; (2) not including an N-glycosylation site; and (3)
 35 hydrophilic (e.g., solvent exposed).

Antibodies were generated in New Zealand white rabbits over a 10-week period. The immunogen includes KLH-peptide emulsified by mixing with an equal volume of Freund's Adjuvant, and injected into three subcutaneous dorsal sites, for a total of 0.1mg peptide per immunization. Animals were bled from the articular artery.

5 The blood was allowed to clot and the serum collected by centrifugation. The serum is stored at -20°C.

For purification, peptide antigens were immobilized on an activated support. Antisera was passed through the sera column and then washed. Specific antibodies were eluted *via* a pH gradient, collected and stored in a borate buffer (0.125M total

10 borate) at ~0.25mg/ml. The anti-peptide titers were determined using ELISA methodology with free peptide bound in solid phase (1µg/well). Detection was obtained using biotinylated anti-rabbit IgG, HRP-SA conjugate, and ABTS.

All antibodies performed well in ELISA assays. Anti-peptide #44, #46, and #58 are particularly useful for detection of hDkk-3 and hSoggy-1, respectively, as

15 determined by western blotting of supernatants from hDkk-3- and hSoggy-1-transfected 293T cells.

The Dkk family comprises a novel family of secreted proteins which to date

20 includes hDkk-1, hDkk-2, hDkk-3, hDkk-4, xDkk-1, mDkk-1 and cDkk-1. Structurally, Dkks 1-4 are related by several conserved features. Firstly, all four proteins are secreted proteins. Secondly, Dkks 1-4 each possess two distinct cysteine rich domains. Each domain contains 10 conserved cysteine residues, and these domains are highly conserved between family members. The C-terminal cysteine rich

25 domain, referred to as CRD-2, bears significant similarity to proteins of the colipase family and sequence conservation among the Dkks is greatest within CRD-2 (Aravind and Koonin, *supra*). This may reflect a need for Dkks to interact with lipids in order to regulate Wnt function, since Wnt proteins remain tightly associated with the cell surface.

30 Despite the similarities between Dkks 1-4, notable differences between these family members appear with regard to their mRNA expression patterns. In adult human tissues hDkk-1 and hDkk-4 showed highly restricted mRNA expression patterns while hDkk-2 and hDkk-3 are more widely expressed. Murine Dkk-3 mRNA was found to be restricted to the myocytes of the atria in the heart, neurons of the

35 cortex and hippocampus in the brain and also to the retinal neurons and lens epithelium in the eye. Such specific expression patterns reflect localized action of the Dkks as regulators of Wnt activity and/or that of other signaling molecules. Different

Wnt family members have been shown to have divergent patterns of mRNA expression in adult and embryonic mammalian tissues. For example, murine Wnts 4, 7a and 7b are expressed in brain and lung, whereas Wnt6 is highly expressed in testis (Gavin *et al.*, 1990). Wnts 5b and 13 are more broadly expressed (Gavin *et al.* (1990) *supra*; Katoh *et al.* (1996) *supra*). Although Wnts have been studied mostly in the context of their roles in embryonic development and tumorigenesis, the expression of many family members in normal adult human and mouse tissues, together with their regulators such as the Dkks, suggests that these signaling proteins play important roles in normal tissue homeostasis.

10 Marked differences in the post-translational processing of different human Dkk proteins was also observed. hDkk-3 is secreted from 293T cells as a heterogeneously glycosylated protein, whereas Dkk-1, 2 and 4 proteins show no evidence of glycosylation. This is consistent with sequence analysis that identifies 4 potential sites of N-glycosylation in the hDkk-3 protein but no sites in either hDkk-2 or hDkk-4. A single putative site in hDkk-1 does not appear to be utilized in 293T cells and may well not be a significant site for N-linked carbohydrate addition in view of its C-terminal location and lack of conservation with xDkk-1. C-terminal proteolysis of hDkk4 in 293T cells was also characterized. Dkk proteins contain multiple potential sites of proteolytic processing. Secreted hDkk-4 was consistently detected as three major C-terminal fragments. N-terminal sequencing identified two of these as mature, full length hDkk4 and CRD-2, which was derived from the full length protein by a specific proteolytic event at lysines 132 and 133. Thus, the hDkk-4 CRD-2 is released from the full length protein upon expression in 293T cells. Similar processing of hDkk4 in COS cells has been observed.

25 Within the Dkk family, Dkks 1, 2 and 4 display closest similarity whereas Dkk-3 is more distantly related. For example, Dkk-3 contains a linker region connecting CRD-1 and CRD-2 which is shorter than in other Dkks. Dkk-3 also possesses extended N- and C-terminal regions compared to other Dkks. Within the Dkk-3 N-terminal unique region, a distinct domain has been identified that is also found in Soggy (the SGY domain). The SGY domains of human and mouse Soggy-1 and Dkk-3 proteins contain two conserved sites of N-linked glycosylation. Protein expression studies confirm that, like hDkk3, hSoggy is secreted as a glycoprotein. Murine Soggy-1 is highly expressed in adult testis and also displays a highly restricted mRNA expression in E15 -E16 mouse embryos, being localized mainly to the cartilage primordia within the developing vertebrae/developing dorsal root ganglia. Soggy mRNA was also detected at high levels in the developing eye, similar to Dkk-3. This

